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EXPERIMENTAL MEDICINE

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SIMON FLEXNER, M.D.

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MEGACARYOCYTES IN THE PERIPHERAL CIRCULATION.*

By GEORGE R. MINOT, M.D.

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PLATE 1.

(Received for publication, December 30, 1921.)

Immature forms of red cells and white cells which originate in the bone marrow are commonly observed in the peripheral blood in many pathological conditions. The presence of large numbers of such cells in the blood is a feature of myelogenous leucemia. It might be expected that similarly either true immature forms, or what correspond to immature forms, of the third formed element of the blood, which also originates from the bone marrow, namely the blood platelets, would be present under those conditions in which we find immature red and white cells.

The megacaryocyte (megalocaryocyte) of the bone marrow has been conclusively shown by Wright (1) to be a parent cell of the platelets. The appearance of megacaryocytes in the peripheral blood does not seem to have been noted in the English literature. Isolated observations that they do occur are reported in foreign literature by Oelhafen (2), Di Guglielmo (3), Cesaris-Demel (4), Kaznelson (5), and Nägeli (6). More recently an article has been published by Pianese (7) and another by Di Guglielmo (8) that also refer to the occurrence of megacaryocytes in the circulating blood. These authors found them in cases of myelogenous leucemia, polycythemia vera, and rarely with simple leucocytosis.

It is the object of this report to emphasize that megacaryocytes occur in the peripheral blood and that they occasionally appear in

* This paper is No. 27 of a series of studies on the physiology and pathology of the blood, from the Harvard Medical School and allied hospitals, a part of the expense of which has been defrayed from a grant from the Proctor Fund of the Harvard Medical School for the Study of Chronic Diseases.

large numbers. I have frequently found these cells in the blood of myelogeneous leukemia patients, and have observed them in two cases of polycythemia vera, one of lobar pneumonia, one of Hodgkin's disease, and one of sepsis.

Owing to the normally large size of the megacaryocytes, one can conceive that if they left the bone marrow they would be unable to pass through the finer capillaries of the lungs. There is ample proof, originally given by Aschoff (9) and since by Bunting (10), MacCallum (11), and Wright (1) among others, that megacaryocytes may become packed in the lung capillaries in various conditions calling forth abnormal or immature bone marrow elements.

The presence in the peripheral blood of immature megacaryocyte elements, namely undifferentiated bits of their cytoplasm, together with large numbers of platelets and the subsequent demonstration of megacaryocytes in the lung capillaries, has been noted in Hodgkin's disease by Bunting (10). Cytoplasmic bits of these bone marrow giant cells, which are misnamed giant platelets by Fonio (12) occasionally have been observed in the circulating blood in various other diseases. The presence of these cytoplasmic bits was associated with marked increase of the blood platelets. Variation in the histology of the platelets has received very little attention as compared with that given to the red cells and white cells. Rather marked pathological variation in the size, contour, and character of the granules of the platelets occurs, as shown in studying them in fresh vitally stained as well as fixed preparations. Some such changes in the platelets may be interpreted as indicating immaturity. I have observed frequently abnormal forms and found bits of megacaryocyte cytoplasm in the blood under a wide variety of conditions, including Hodgkin's disease, but notably in myelogenous leukemia. These abnormal derivatives of the megacaryocytes are seen particularly when there is a considerable increase of platelets. The more marked deviations from normal occur when there is other evidence of intense activity of a pathological marrow.

The character of the megacaryocytes seen in the peripheral blood is shown in the accompanying plate.¹ These giant cells have

¹ It is a pleasure to acknowledge that Dr. J. H. Wright has examined some of the preparations from which these cells were drawn and that he considers them to be identical with megacaryocytes seen in the marrow.

distinctive characters more readily observed than described. In the bone marrow the megacaryocyte often characteristically develops a large amount of cytoplasm with ameboid activity so that it projects pseudopods into the blood channels. The formation of platelets results from the segmentation of the pseudopods. Pseudopods not connected with a nucleus are seen not infrequently and, as indicated above, may enter the blood stream. The cytoplasm when stained with Wright's stain contains numerous characteristic red to purple granules, and its edges often appear to be hyaline. Vacuoles occur in the cytoplasm. In the marrow the amount of cytoplasm associated with the megacaryocyte nucleus varies in consequence of the formation of platelets, and nuclei showing the usual signs of degeneration with little or no cytoplasm are frequent. In the peripheral blood the nuclei appear usually to be stripped of their cytoplasm, but as shown in the plate some retain a small amount, which is in the process of differentiation into platelets. In some instances formed platelets may become adherent to the nuclei when the preparation is made. In the bone marrow the nucleus of these cells often appears as a complicated pleomorphic structure that typically is lobate and basket-shaped. Owing to its character the nucleus may appear multiple; if it is actually multiple, this indicates degeneration. The nucleus in the marrow is frequently more vesicular than when seen in the blood stream. However, these nuclei seen in the peripheral blood are entirely similar to many of those seen in the marrow. They are of varying shapes and sizes, stain deeply, and present the characteristic spotty internal structure shown in the drawings. At times only a fragment of a nucleus occurs. Many of the nuclei seen in the peripheral blood are smaller than the majority of those seen in the marrow. At autopsy of three cases of myelogenous leucemia there were demonstrated in the lungs many much larger megacaryocytes than were found in the peripheral blood during the last days of life. The lungs of these individuals presented a remarkable appearance. The alveolar walls were thickened and many of the capillaries were plugged with enormous numbers of megacaryocytes. It would seem as if this capillary embolism might have embarrassed the pulmonary circulation and contributed to the dyspnea that these patients had during the last few days of

their life. However, they all had extreme anemia, which could account for their dyspnea. With an increased formation of megacaryocytes diminutive and atypical forms of megacaryocytes are frequently seen in the marrow. Increased numbers and diminutive and atypical forms are characteristic of myelogenous leukemia. This is in harmony with the conception that cells of tumors and tumor-like processes deviate from type and show atypicality. Thus it is not surprising that small megacaryocytes that can pass through the lungs occur in the blood of these cases. Many of the megacaryocyte nuclei observed in the peripheral blood are small, due to fragmentation in consequence of degeneration.

It is not at all uncommon to find an occasional small megacaryocyte (less than 1 per cent of the white count) in the peripheral blood in chronic myelogenous leukemia. During the continued study of some forty-five cases of this disease I have observed at one time or another megacaryocytes in thirty-five cases. In some instances these cells were observed before any form of radiation was given the patient, in others after such therapy. Certain cases showed these cells more persistently than others. When an occasional megacaryocyte was observed the patient was not so well as when they were absent and the white count was usually over 50,000 per c. mm. Even so, many patients showing an occasional one of these giant cells were not particularly sick. In six cases the megacaryocytes occurred in relatively large numbers. Two individuals, one who had had the disease for 4 years and the other for 6 years, showed from 1 to 8 per cent of these cells in white counts of from 50,000 to 150,000 per c.mm. during their last 3 months of life. Three others showed 1 to 4 per cent of these cells in white counts of from 75,000 to 400,000 per c.mm. All five of these cases were sicker than many of those who had an occasional megacaryocyte in their blood. They showed other evidence of unusual activity of the disease at the time the megacaryocytes were present as demonstrated by the presence of fever, a basal metabolism in the vicinity of +60, and numerous myeloblasts and other immature bone marrow cells in the blood stream. The sixth case that showed many of these giant cells was a most unusual one. The patient, who did not appear particularly sick, was a woman of 45 years, with an enormous spleen

and the symptoms of leucemia; before radium treatment she showed a hemoglobin of 75 per cent and a white count of 40,000 per c.mm. Many of these elements in carefully made preparations were broken, indicating their immaturity, and they could not be identified. The striking feature of the differential count of the intact cells was that 18 per cent were megacaryocytes and but 16 per cent myelocytes of various types. Myeloblasts were rare. The remaining cells were largely polynuclear neutrophils. Nucleated red cells were observed occasionally. The platelets were greatly increased. The patient, though continuing to have a large spleen, has been symptomatically very definitely benefited in the past 2 years by radium therapy. During this time the blood has shown no higher white count than 40,000 per c.mm., and seldom more than an occasional megacaryocyte, usually none. The immature white cells have fluctuated considerably in number, as have the platelets.

In two cases of typical polycythemia vera, one of Hodgkin's disease, one fatal case of lobar pneumonia, and one of sepsis, I have found an occasional, usually small, megacaryocyte. The blood in all of these cases showed increases of platelets with bits of megacaryocyte cytoplasm. The two cases of polycythemia were not particularly sick. Both showed various early forms of nucleated red cells and white counts of 25,000 to 40,000 cells per c.mm.; although no undoubted myeloblasts were present, 4 to 8 per cent of myelocytes occurred. The patients with pneumonia and sepsis both had a white count of about 90,000, consisting chiefly of polynuclears, but with a few myelocytes and normoblasts. The case of Hodgkin's disease, that died shortly after megacaryocytes were found in the blood, had a white count of 40,000. The polynuclears were 80 per cent, the large mononuclears 19 per cent, and the eosinophils 1 per cent, and a few normoblasts were seen.

Increase of platelets was the rule when megacaryocytes were found in the blood, but in some instances of myelogenous leucemia megacaryocytes were present when the platelets occurred in normal numbers and rarely when they were decreased. In all the instances in which more than an occasional megacaryocyte was found, there was a marked and usually an extreme increase of platelets, reaching to

over 3 millions in one case. In such cases bits of megacaryocyte cytoplasm were found frequently. When the giant cells were found there was usually other evidence of a serious alteration in the rate of emergence of the cells from the marrow as shown by the presence of many immature cells of both the white and red series, though the white count was not necessarily very high or the anemia marked. In myelogenous leucemia with the white count below 30,000, myelocytes less than 12 per cent, and no myeloblasts, the appearance of megacaryocytes and increases of platelets may be the first sign of increased activity of the disease, as shown in two cases. Usually myeloblasts and primordial nucleated red cells were found, in the cases of myelogenous leucemia when megacaryocytes were present. Of course, as is well known, myeloblasts and primordial nucleated red cells occur without megacaryocytes. None of these three elements normally occurs in the blood stream. Their presence in the blood stream is sufficient to indicate a serious alteration of the regulatory mechanism for the emergence of cells from the bone marrow into the circulating blood. The marrow in such instances is subject to great strain. The pathological process which underlies this altered regulatory mechanism is varied, since these immature cells occur in the blood not only when there are structural changes in the bone marrow, as in myelogenous leucemia, but also with other changes, probably largely functional, as in pneumonia and sepsis.

Apparently when megacaryocytes appear in considerable numbers in the circulating blood, the pathological process in the marrow particularly involves this element. In general the presence of these cells in the blood stream is not to be looked upon as indicative of a desirable type of marrow regeneration but more as indicative of a marrow disintegration, recovery from which may, however, occur.

CONCLUSIONS.

A megacaryocyte is seen commonly as an occasional cell in the peripheral blood of patients with myelogenous leucemia. Less commonly they appear in relatively large numbers.

These giant cells also may occur in the blood under other conditions.

Their presence is indicative of a bone marrow under intense strain.

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EXPLANATION OF PLATE 1.

This plate illustrates selected megacaryocytes found in ordinary cover-glass blood smear preparations of the peripheral blood from six different cases. The preparations were stained with Wright's modification of Romanowsky's stain. The drawings were made by the aid of a camera lucida. $\times 1,300$.

The seven megacaryocytes labelled 1 are from a case of myelogenous leucemia. Some are naked nuclei. The larger uppermost nucleus shows some adherent cytoplasm of a megacaryocyte.

The groups of cells labelled 2, 5, and 7 are also from cases of myelogenous leucemia.

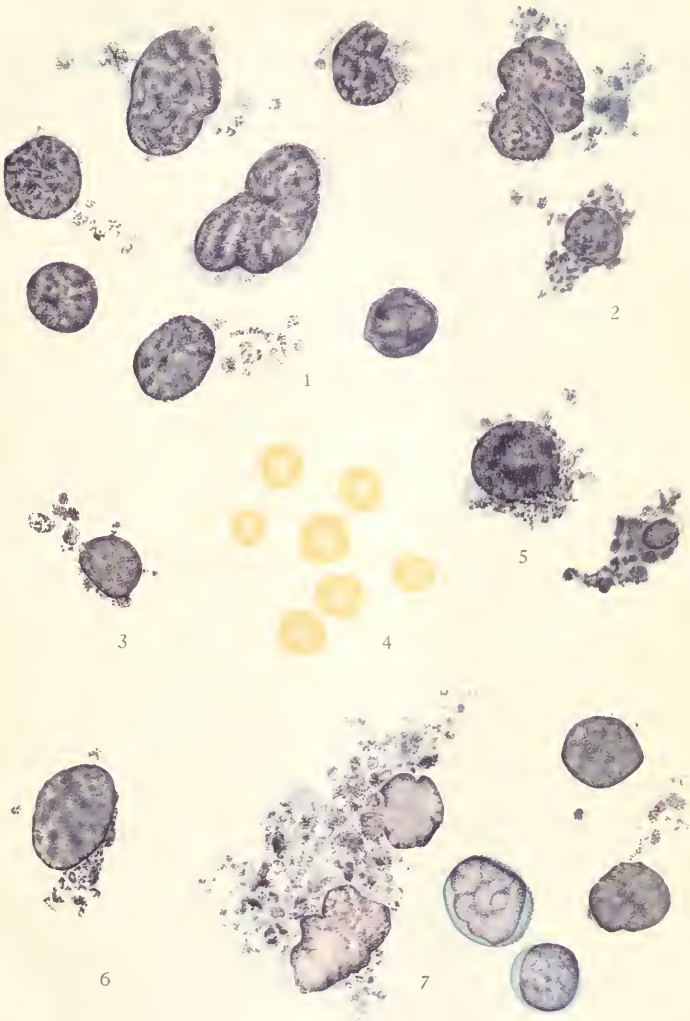
In Group 2 megacaryocyte cytoplasm with its differentiation into platelets is distinct.

In Group 5 is shown a fragment of megacaryocyte nucleus with cytoplasm; differentiation into platelets is taking place.

In Group 7 two myeloblasts (the two central cells) are shown for contrast. On the left the large mass of platelets and megacaryocyte cytoplasm, associated with the somewhat degenerated megacaryocyte nuclei, may have come into approximation with the nuclei in the process of making the preparation.

The small megacaryocyte in Group 3 occurred in a case of lobar pneumonia, while that shown in Group 6 is from the blood of a case of polycythemia vera.

The red cells (Group 4) are inserted to illustrate the comparative size of the megacaryocytes.



EXPERIMENTAL EPIDEMIOLOGY.

INTRODUCTORY.

By SIMON FLEXNER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 15, 1922.)

Each generation receives its particular impression of epidemic diseases; during the past 20 years the impressive epidemics of the western world have been those of meningitis or cerebrospinal fever, poliomyelitis, influenza, and latterly lethargic or epidemic encephalitis. These several epidemic diseases have numbered their cases by the hundreds, thousands, and even tens of thousands in given communities or countries, and have ravaged not only the United States, but within the same period have prevailed, sometimes with more, sometimes with less intensity, in other and distant parts of the world.

All these epidemics are indeed old-world diseases, and excepting the last, namely lethargic encephalitis, regarding which knowledge is still scant and uncertain, they can be traced far back in recorded human history. Moreover, the remarkably wide or pandemic outbreaks of which the recent American epidemics form part all originated in the old world and were communicated, usually after a lapse of time, to the new world, where, in some instances, as in the notable one of the 1916 wave of poliomyelitis, they found a soil so fertile and circumstances of spread so favorable as to reach a height of destructiveness previously unknown.

Ever since Hippocrates and especially since Sydenham, the study of epidemics of disease with a view to penetrating their hidden meaning has engaged the attention of occasional men. The degree of interest in what may be called the nature of epidemics has, however, fluctuated greatly and considerable periods have elapsed in which the subject has been given only superficial thought. Then

circumstances have arisen through which both the professional and the public mind has become so engrossed with it that new efforts come to be put forth in order to grasp its significance.

We are moving now in such a period of revived interest. Ever since the rise of modern bacteriology, the hope has been indulged that as knowledge of the microbic incitants of disease grew, the nature of epidemics would become more comprehensible. That this hope has not been entirely disappointed is shown by the influence which precise knowledge of certain disease-producing microorganisms, taken directly into the gastrointestinal tract or communicated by insect vectors, has had on the control of maladies thus induced. The discoveries in these two fields alone and the measures derived from them, as well as the discovery of the part played by those potential distributors of the microbes of disease called "carriers," have led to practical achievements of great magnitude in the domain of hygiene, imparting a sense of human power and control over impending epidemics of disease, in profound contrast with the helplessness of any previous period in history.

And yet in certain quarters, and especially in England, what may be defined as a reaction against the teachings of modern, that is bacteriological, epidemiology seems to have set in. That this return to older, and as it must seem, more mystical doctrines is widespread cannot be affirmed, but it has even happened that in the effort to elucidate the epidemic prevalences of the past two decades already enumerated and eventuating in the colossal outbreak of influenza, the notions of epidemic constitutions as defined by Hippocrates and especially by Sydenham have been more or less sported with, if not actually invoked.¹

¹ Payne, J. F., Thomas Sydenham (Masters of medicine series), London, 1900. Greenwood, M., Sydenham as an epidemiologist, *Proc. Roy. Soc. Med.*, 1918-19, xii, Epidemiol. and state med. sect., 55. Goodall, E. W., Discussion on Sydenham, *Proc. Roy. Soc. Med.*, 1918-19, xii, Epidemiol. and state med. sect., 66. Singer, C., Discussion on Sydenham, *Proc. Roy. Soc. Med.*, 1918-19, xii, Epidemiol. and state med. sect., 71. Greenwood, M., The factors that determine the rise, spread and degree of severity of epidemic diseases, *XVII Internat. Cong. Med.*, 1913, sect. 18, 49. Report on the pandemic of influenza, 1918-19, *Ministry of Health, Rep. Pub. Health and Med. Subj.*, No. 4, 1920.

To the extent to which this reaction is an endeavor to define epidemiology in terms wider than those of the microbic incitants of disease alone it may be regarded as wholesome and timely. The doctrine of "carriers" of potentially infecting microorganisms necessitates a conception of epidemic disease wider than is embraced in the exclusive view of their varying pathogenic activities. That the mere occurrence of potent microorganisms does not suffice to produce an outbreak of epidemic disease is a commonplace of bacteriological knowledge. The conditions are not as simple as that, and in their greater complexity they include not only the various qualities of the microbe but also of the host and, as well, their many reactions, one upon the other.

But it may be questioned whether progress is to be gained by a return to the indefinite concepts of epidemic constitutions or of the interdependence of diseases as various as epidemic meningitis, poliomyelitis, influenza, etc., united by an inevitable nexus of events the nature of which cannot even be surmised.²

Against these vague considerations modern bacteriology opposes the belief in a specific etiology, in which a particular microorganism is sought as the incitant of the infectious disease under study. The triumphs already secured in this field need not be recited here. But it is obvious that the gains made are not sufficient to account for all the phenomena of epidemics. The search, therefore, must, if possible, be both widened and deepened. Hence, the immediate question which presents itself is the manner in which this can be done.

² Hamer, W. H., The Milroy lectures on epidemic disease in England—the evidence of variability and of persistency of type, *Lancet*, 1906, ii, 569, 655, 733; Discussion on influenza, *Proc. Roy. Soc. Med.*, 1918–19, xii, Roy. Soc. Med. sect., 24; Discussion on Sydenham as epidemiologist, *Proc. Roy. Soc. Med.*, 1918–19, xii, Epidemiol. and state med. sect., 72. Newsholme, A., and Stevenson, T. H. C., et al., Discussion on influenza, *Proc. Roy. Soc. Med.*, 1918–19, xii, Roy. Soc. Med. sect., 1. Crookshank, F. G., First principles: and epidemiology, *Proc. Roy. Soc. Med.*, 1920, xiii, Epidemiol. and state med. sect., 159; A note on the history of epidemic encephalomyelitis, *Boston Med. and Surg. J.*, 1920, clxxxii, 34; Public health considerations relating to influenza, pneumonia, and allied epidemics. The epidemiological point of view, *Boston Med. and Surg. J.*, 1921, clxxxiv, 548. Report on the pandemic of influenza, 1918–19, *Ministry of Health, Rep. Pub. Health and Med. Subj.*, No. 4, 1920.

Epidemic outbursts of disease occur among animals and pursue a course similar to that which has been observed to occur in man. The direct study of epidemics among animals under conditions of control not attainable in man should therefore commend itself to the epidemiologist. By this means it may be possible to secure those precise data of both microorganism and host on which eventually a real science of epidemiology may come to be built.

Thus an investigation was undertaken several years ago relating to certain aspects of experimental epidemiology of which the papers to follow are the first fruits. The papers relate to epidemics in mice of gastrointestinal origin, to which the name of mouse typhoid is applied. While mouse typhoid presents clinical and pathological characteristics of a single disease-complex, its microbic incitant is not a consistent species. Indeed, just as there are distinct but related bacilli inducing dysentery in man, there are distinct but related bacilli capable of provoking "typhoid" in mice. This relative multiplicity of microbic factors may be viewed as an aid rather than as a hindrance in investigating the events taking place in course of epidemics, the knowledge of which may come to have applicability to man as well as to the lower animals. The several bacilli alluded to differ little in cultural, but profoundly in immunologic properties. Some of the species exist either as saprophytes or at least as harmless "carried" bacteria in mice regarded as normal and yet become substituted for the original inciting microbes during epidemics—a fact of high importance in respect to the vagaries noted in the bacteriologic investigations of certain epidemics in man.

Not only do specific differences exist among so called mouse typhoid bacilli, but all the pathogenic varieties appear extremely labile. Strains of the bacilli artificially enhanced as they pass from mice to mice quickly fall to an average of infectivity and are, as it seems, at low pathogenic ebb at the time of the death of the infected animals. But this lability of the bacilli is determined, in part, by the hosts; that is, the mice through which they pass. In this respect mice may be viewed as consisting of different biological classes according as they respond to ingestion of the bacilli with infection and death, with mere carriage of the bacilli, or with non-reactibility. The distinctions of classes are not, however, absolute, but are determined, partly at

least, by the quantity or dosage of the bacilli. It is this latter factor which plays so conspicuous a rôle in the phenomenon of recurrent epidemic waves superinduced by the introduction of new mice in the replacement experiments described.³ While it is the "carrier" among the old mice which provides the "seed" for the next following epidemic outbursts, it is the highly susceptible individuals among the new which furnish the living "culture" medium enabling rapid increase and wide dissemination of the bacilli to be effected, just as it is the succumbing and non-reactible mice which check the growth and multiplication that tend to arrest the epidemic spread.

These factors leave out of account the effect, if any, of active immunization acquired through previous exposure, a condition shown experimentally to be realizable, but the extent and sufficiency of which in initiating the events and finally in bringing about the state of equilibrium between parasite and host prevailing at the end of the epidemic wave are still undetermined.⁴

The papers in this series relate to a gastrointestinal infection in mice. In man the prevalent type of epidemic disease has altered notably within a century; there has been far less of the diseases in which the mode of infection is enteric and more of the diseases in which the portal of infection is by way of the respiratory tract, which fact leads to the consideration that the control which modern hygienic practises exercise over epidemics depends on the imposition of general or communal, as opposed to individualistic measures of prevention. Smallpox is controlled by essentially universal vaccination; typhoid and allied fevers by water purification and similar means; malaria, typhus, and yellow fever by war on their insect propagators. While conversely the epidemic diseases which still prevail almost unchecked among the western peoples are just those for which no communal means, practically applicable, exist of preventing communication of the potentially morbid materials, such as those of the secretions of the respiratory tract, from individual to individual. And coinci-

³ Amoss, H. L., Experimental epidemiology. II. Effect of the addition of healthy mice to a population suffering from mouse typhoid, *J. Exp. Med.*, 1922, xxxvi, 45.

⁴ Webster, L. T., Experiments on normal and immune mice with a bacillus of mouse typhoid, *J. Exp. Med.*, 1922, xxxvi, 71.

dentally with this circumstance, other significant conditions have arisen to modify previous history, namely the rise of multitudinous cities, industrialism, rapid transport, etc., the effects of which are to increase the number and intensity of personal contact associations, and thus to combine, confuse, and distribute quickly and widely the respiratory secretions of unnumbered persons.

It is our intention to extend the experimental investigation of epidemics among laboratory animals to the respiratory-borne infections. Opportunities for such studies undoubtedly exist. The obvious differences and inconsistencies between the animals thus affected and man, primarily in respect to the distinction in habits, may not prove insuperable obstacles in the way of obtaining illuminating information from the one applicable to the other. In any case it will be found desirable to check the experimental studies on epidemics of enteric origin with those of respiratory origin and to determine in how far they pursue similar and to what extent they follow different modes of evolution and devolution.

AN OUTBREAK OF MOUSE TYPHOID AND ITS ATTEMPTED CONTROL BY VACCINATION.

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In this paper will be described briefly an epidemic of so called mouse typhoid which prevailed with fluctuations for approximately $2\frac{1}{2}$ years in a mouse breeding station maintained at The Rockefeller Institute.

The original stock of about 3,000 mice of all ages was procured by purchase and transported *en masse* to The Rockefeller Institute. The stock was especially valuable because it yielded a relatively high percentage of mice developing so called spontaneous cancers, located in the mammary glands chiefly, but also in other organs. It is known that the original stock, from which that purchased by The Rockefeller Institute was derived, had previously suffered losses from mouse typhoid. Moreover, to the stock as purchased, there were added from time to time from the outside a mouse showing a spontaneous tumor or other small accessions of healthy stock.

The transfer of the mouse stock took place in April, 1918. Nothing especially noteworthy in the fatalities arose to arrest attention until about the middle of September of that year, when an unusual number of deaths among the stock occurred.¹ From this time on, until what may be called the termination of the epidemic period, some $2\frac{1}{2}$ years later, the number of deaths may be regarded as having been abnormally high, although in intervals between the wave-crests, the death rate returned to a level as low as that usually regarded as normal.

It was customary to record all deaths occurring in the stock mice and at the beginning of each month to take a census of the population

¹The summer death rate immediately preceding the September rise was slightly higher than usual. But as during the warm summer period previously a rise had been observed, this increase was not considered significant.

which fluctuated in number roughly between 2,500 and 4,000 individuals. The numbers as given by the census varied according to the matings and to the demands made on the stock for purposes of investigation.

It was the custom also to make a gross postmortem examination of every mouse found dead. When the deaths became so frequent as to excite apprehension of an impending epidemic, bacteriological studies were made. Cultures of the spleen and liver yielded a bacillus which was identified as belonging to the group of mouse typhoid bacilli. During the course of the bacteriological studies about a dozen strains conforming to the cultural characteristics of the group were isolated. Of this number two strains were retained in culture and transplanted regularly and thus kept alive. But it was not until about a year after the epidemic began that the two strains were subjected to immunological study by Dr. Amoss.

The two strains proved to be identical in cultural and immunological reactions, and there was no reason to suppose that the first epidemic was induced by more than this one strain of a bacillus belonging to the enteritidis group. As this strain was utilized by Dr. Amoss in certain of his experiments on the production of artificial epidemics among mice,² its more precise biological description will be found in his papers.³ For sake of convenience the strain has been designated Mouse Typhoid I.

Before proceeding to the more detailed account of the epidemic among the stock of cancer mice, as they were called, a brief statement regarding the conditions under which mice are propagated at The Rockefeller Institute may be of interest.

Two distinct breeding rooms or stations for mice are maintained, with separate caretakers who do not mingle. One station houses the cancer stock and the other the normal stock. The latter has been inbred for the past 2 years, no outside accessions having been made during this period, and is employed in the general investigative work of the Institute. The completeness with which separation between the two stations has been effected is indicated by the fact that in the long period during which deaths—many or few—from mouse

² Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 25.

³ Amoss, H. L., and Haselbauer, P. P., *J. Exp. Med.*, 1922, xxxvi, 107.

typhoid were taking place among the cancer stock there were only four deaths among the normal stock apparently from this cause. The population of the normal stock was kept approximately at from 2,500 to 3,000 individuals.

The Epidemic.

The course of the epidemic outbreak can best be followed by observing the graphic curve which is reproduced in two sections which have been subdivided into segments (Text-fig. 1).

As the first segment (No. 1) of the curve indicates, the death rate among the mice rose in the 2nd week of September and actually became about twice the usual weekly rate. From this time on the records were kept more accurately than before and they show a gradual rise in the number of deaths up to the 1st week in November, when the rate remained constant for 10 days, increased during the 3rd week, and began to decline abruptly.

The curve indicating actual number of deaths forms a plateau extending over a period of 2 weeks during which there was a slight depression. The decline was slightly more irregular than the rise.

The course of the epidemic is perhaps better described by the attack rate per 1,000 of population, which rose sharply until November 19 and declined as rapidly as it had risen.

The return to what is regarded as the usual death rate came about January 18, 1919. The outbreak lasted about 140 days; the peak was reached at about the middle of this period, or the 80th day (November 19), so that the curve representing the rate per 1,000 is fairly symmetrical with a slight lag in the decline.

From a consideration of the character of the curve during December, there is a suggestion of the occurrence of another small wave which might have begun somewhere between December 15 and 20 and reached its peak on December 29. The interval between the peaks is about 15 days. Such a supposition is strengthened by the occurrence of a similar wave in March (shown in Segment 2) which endured for 15 days. It is not improbable that the total outbreak is really a series of six overlapping epidemic waves, each lasting about 15 days. Unfortunately no spot map was kept recording the distribution of the deaths in the breeding station. In general the epi-

TEXT-FIG. 1. Segment 1. Deaths from all causes among the cancer breeding stock by 5 day periods from September 5, 1918, to January 18, 1919.

Segment 2. Deaths from all causes by 5 day periods from January 19 to April 30, 1919.

Segment 3. Deaths from all causes by 5 day periods from May 1 to September 30, 1919.

Segment 4. Deaths from all causes by 5 day periods from October 1, 1919, to February 29, 1920.

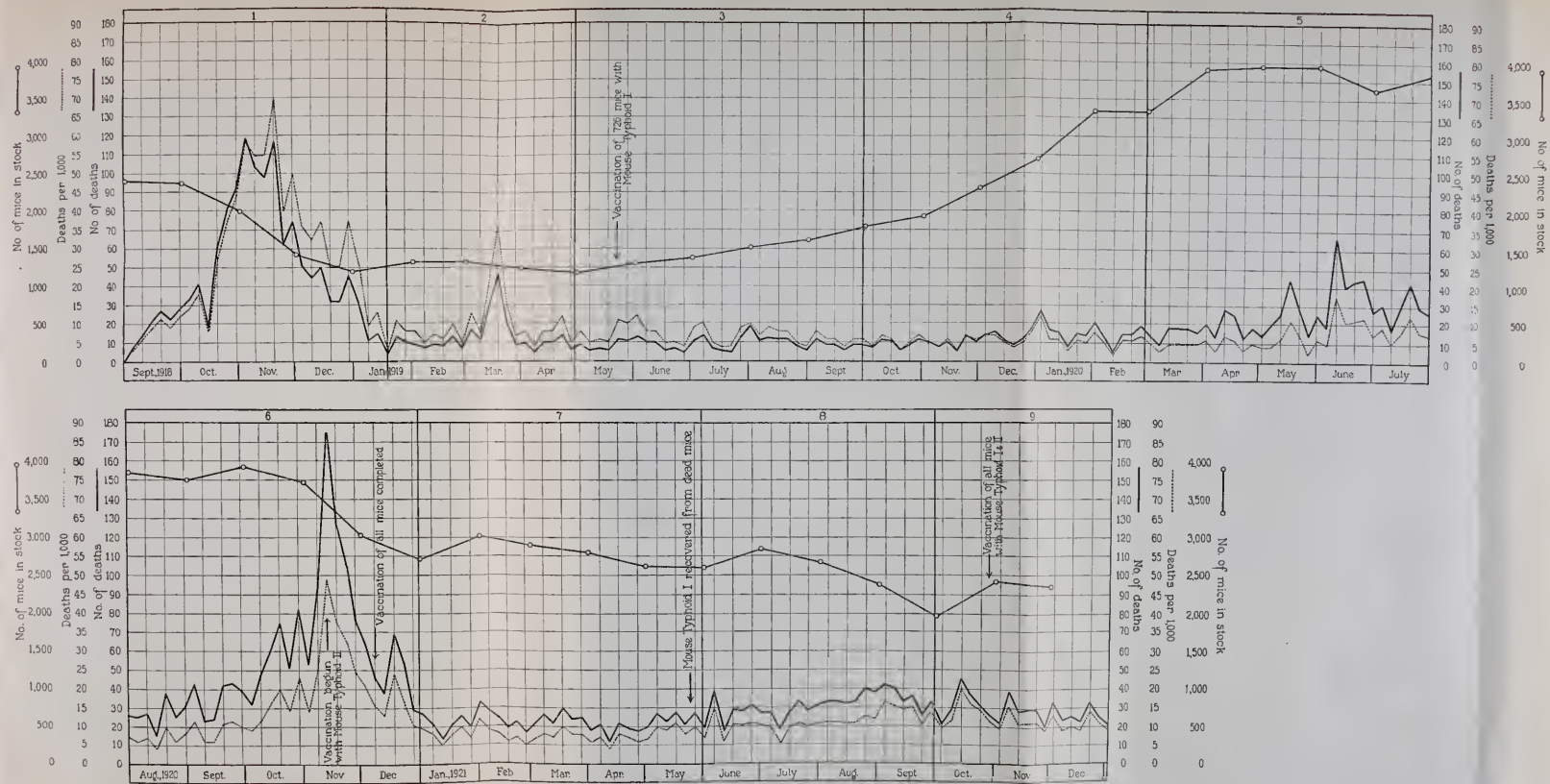
Segment 5. Deaths from all causes by 5 day periods from March 1 to July 31, 1920.

Segment 6. Deaths from all causes by 5 day periods from August 1 to December 31, 1920.

Segment 7. Deaths from all causes by 5 day periods from January 1 to May 31, 1921.

Segment 8. Deaths from all causes by 5 day periods from June 1 to September 30, 1921.

Segment 9. Deaths from all causes by 5 day periods from October 1 to December 31, 1921.



TEXT-FIG. 1Deaths per 1,000 of mouse population by 5 day periods. ——— Actual number of deaths in mouse population by 5 day periods. ——— Total number of mice in stock according to the monthly census.

demic raged on the shelves where the older mice were kept. For example, among 1,465 older mice the mortality was 82 per cent, whereas among 1,034 new mice, most of which were weaned before the onset of the epidemic, the mortality was 43 per cent.

The severity of this first, or as it may be called November epidemic wave, is shown by the number of deaths presumably in all instances from mouse typhoid. This number as calculated for the period from September 10 to January 28 inclusive is 1,351 mice, or a little more than one-half of the population of the breeding station. During December the deaths occurred mainly in boxes which had already been attacked. Instances of spread to new boxes were frequent.

It is quite certain that this large number of deaths was brought about by the mouse typhoid infection, for while not every dead mouse was studied bacteriologically, yet all were submitted to autopsy and the usually obvious signs of the disease detected.

Along with the epidemic there occurred as a concomitant effect a large reduction in the birth rate so that the number of mice diminished through death was not augmented by births. Hence during the next period of January, February, and 1st week in March, as shown by the second segment (No. 2) the death rate, while still high as compared with the pre-epidemic period, kept quite uniform. However, during the 1st week of March an upward tendency is evident, which at the beginning of the 2nd week is converted into a steady rise culminating in a peak lower than the November peak, enduring a shorter time, and falling more sharply, as the average death rate is again reached in the 1st week of April.

This second, or March epidemic wave is to be considered in the light of two classes, as it were, of the mouse population; namely, the old population which had passed through the November epidemic, and the accessions through new births. The total population had been profoundly reduced by the November epidemic and not yet restored by new births. The March wave was of short duration (15 to 20 days) and was approximately half as severe as that of November. As already stated, the November outbreak may be looked upon as a series of waves following closely one another, whereas the March epidemic represented a single wave.

A period of relative quiescence now set in, as shown by Segment 3. On May 17 the total mouse population was about 1,250 individuals. During the next 4 weeks, half of the mice were given (vaccinated) under the skin of the back a single injection of 0.2 cc. of a suspension (or about 600,000) of killed bacilli of the strain of mouse typhoid isolated during the epidemic. The vaccination produced no immediate effect on the death rate, although it is noteworthy that during a 4 weeks period of mid-May to mid-June, only two of the vaccinated mice died and in only one was the mouse typhoid bacillus found.

The carrying out of the vaccination would seem rather to have coincided with than to have been responsible for the quiescent period which extended from June, 1919, to about January, 1920, the level of which is shown in Segments 3 and 4. During this period the mouse population was rising steadily chiefly through new births. The new individuals were left with the old and no further vaccination was carried out in the breeding station. Although the general death rate had not fallen to that of the pre-epidemic period, yet the new equilibrium which had been established was regarded as satisfactory.

However, beginning in January, 1920, as the population continued to increase, a small rise in death rate also occurred as is shown in Segments 4 and 5. By May, 1920, the total population in the breeding station had reached approximately 4,000. As presaging of the next epidemic, events may be detected in the rise in number of deaths in April, May, and June, as indicated in Segment 5. Although the population had again become large, this mere numerical increase was followed with some anxiety.

The number of deaths per 1,000 continued generally high during July, August, and September. Beginning the 1st week in October, a further rise took place that with minor and perhaps unimportant fluctuations led into the sharp epidemic wave of November, 1920, which exceeded in actual number of deaths that of the first, or 1918 November wave, but the rate per 1,000 was only five-sevenths of that recorded in the 1918 outbreak. The course of this epidemic wave which was practically obliterated by January, 1921, is shown in Segment 6. The wave-like character of the curve is also present. Whereas the epidemic of 1918 seemed to be a summation of six waves, the 1920 outbreak consists of only four, each of which was longer and lower than in the former.

An analysis of this epidemic wave yields the following data. The total mouse population affected by this epidemic wave during its entire course was 4,282, of which 1,463 died. Hence the gross mortality was 34 per cent. Of the 4,282 mice in the total population, 220 were at least 18 months old and had thus passed through the previous severe epidemic wave, while 4,062 were new individuals not so severely exposed. Taking the two classes of the population separately, the old mice (18 months old at least),⁴ and the new mice as above described, it may be stated that of 220 of the former, 130, or 60 per cent, succumbed in the November, 1920, epidemic wave, while of 4,062 of the latter, or new stock, only 1,333, or 32.8 per cent, fell victims. The effect of the epidemic on the birth rate was marked. During the extreme height and the following wave in November and December, very few births took place. By January 1, 1921, the total population had fallen to 2,731 and the sharp outbreak may be regarded as having come to an end. For the succeeding 5 months (January to May) the death rate remained at the average low level ordinarily observed in stocks of mice (Segment 7). The usual slight increase in death rate was observed during the summer (Segment 8) and autumn (Segment 9).

There is practically no difference in the seasonal distribution of the two major outbreaks. Each was preceded by a slightly increased death rate during the summer months and began to gain headway in September, reaching the peak on November 19, 1918, and November 13, 1920, respectively. Since the death rate is calculated on a basis of 5 day periods, the difference of 6 days is probably within the limit of error. The entire duration of the 1918 outbreak was about 140 days and of the 1920 epidemic 125 days.

The bacteriology of the November, 1920, outbreak has particular importance. Practically all the dead mice were examined post-mortem, and cultures of the mouse typhoid bacillus were obtained by Mr. Sturm from approximately 75 per cent of the examined animals. Of the many cultures thus obtained, two strains were retained and eventually turned over to Dr. Amoss for use in his experiments.

⁴ The average longevity of mice in non-infected stock is about 2 years. Old age must be considered as contributory to a high death rate in such a group.

The immunological study by Dr. Amoss of these cultures showed that they differed from the original cultures isolated in the 1918 epidemic. A full description of this bacillus will be found in another paper of this series.³

The fact that the two strains of the paratyphoid-enteritidis group, differing immunologically from each other and both potentially capable of setting up severe epidemics among mice, were responsible for the epidemics separated from each other by 2 years, comes to have a special interest and may possess a particular significance in view of the vaccinations carried out in the period between the two epidemics. For superficially, at least, it appears that the inoculation of the killed cultures of the first bacillus shunted, as it were, that particular organism out of action while leaving the recruited population, both old and new, and the old even more than the new, subject to a second variety of the mouse typhoid bacillus. If this is at all a true statement of what has taken place in the second November epidemic, then the vaccination of part of the surviving population in May and June, 1919, was sufficient to protect the entire population from infection with the first variety of the mouse typhoid bacillus. This last point is obviously one that is open to experimental inquiry as is also the collateral point whether cross-immunity reactions occur between the two varieties of the *Bacillus enteritidis* with which we have been dealing.

No further outbreak of mouse typhoid of an epidemic character has taken place in the cancer breeding station since January, 1921. Just as a large part of the surviving mice was vaccinated after the November, 1918, epidemic wave, so vaccination of all the survivors was carried out at the height of the November, 1920, epidemic wave. The vaccine employed in 1920 was identical with that employed in 1919, and as the two varieties of inciting bacilli differ immunologically, it may be regarded as at least questionable whether the second inoculations have had anything to do with the relative quiescence of the mouse typhoid infection in the breeding station following the last November epidemic.

A slight increase in the death rate occurred during the summer of 1921, and since Mouse Typhoid I had been recovered from some of the mice dying in May the entire population was vaccinated with Mouse Typhoid I and II in October.

Carriers.

No extensive study was made of carriers. In January, 1922, ten mice which had passed through the second epidemic and ten mice born of these were killed and cultured. The cultures were negative for Mouse Typhoid I and Mouse Typhoid II, except for those from one mouse in the former group. In this mouse Mouse Typhoid I was recovered from the cecum only. Cultures from the small intestine were negative for Mouse Typhoid I and Mouse Typhoid II.

EXPERIMENTAL EPIDEMIOLOGY.

I. AN ARTIFICIALLY INDUCED EPIDEMIC OF MOUSE TYPHOID.

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The problem set ourselves was the study of an epidemic of mouse typhoid conducted under experimental conditions. The painstaking observations recorded in Dr. Lynch's paper,¹ far exceeding those usually accorded outbreaks of disease among domesticated animals, seemed to put rather than to answer questions of epidemiology. It appeared to us therefore that an epidemic started under fixed conditions, in which one person followed the happenings day by day and recorded the events, would not only tend to eliminate errors traceable to the elements of surprise and lack of preparation, but also by providing a more homogeneous material yield results of greater consistency, while the two major factors of host and parasite would be placed under highly favorable conditions of control.

Method.

Of laboratory mammals, mice are most easily assembled and observed in large numbers. Moreover, they are subject to a bacterial infection, mouse typhoid, of gastrointestinal origin, the pathology of which is quite well known. The lesions found in and characteristic of the disease affect several important viscera and are obvious to the unaided eye. The bacteriology also is sufficiently worked out so as to serve as a guide in what may be regarded as doubtful instances. Moreover, as is well known, the disease mouse typhoid constitutes a common sporadic fatal epidemic affection among mice and even from time to time sweeps through mice colonies in highly destructive waves.

¹ Lynch, C. J., *J. Exp. Med.*, 1922, xxxvi, 15.

In view of the experiences described in Dr. Lynch's paper,¹ our experimental investigations were started with mouse typhoid. While under way, Topley's² series of valuable papers on the same general topic began to appear in print. They will be discussed along with the deductions from our own observations in the proper place.

The procedure adopted by us at the outset was one chosen to simulate the conditions of epidemic outbreaks of disease not only among carefully segregated small domestic animals but also those which occur among human beings.

Thus what may be termed a mouse village was set up by placing in rows on metal shelves metal cages 7 by 10 by 5 inches with wire mesh tops. 5 mice were placed in each cage without communication between the cages, so that infection could be transferred only by the hands and implements of the person cleaning the cages and feeding the mice. Great care was taken throughout the experiment to exclude roaches, ants, flies, and other insects and vermin from the room. The temperature of the room was kept constantly at 68°F. whenever the outside temperature was below this. In the summer months the temperature of the room was, of course, higher. The cages were thoroughly cleaned once each week, always in the same order, and the mice were fed in the same order daily as follows: grain in the morning and bread moistened with milk in the afternoon. Precautions were taken to exclude extraneous disease. The mice used came from a carefully controlled healthy stock bred in the Institute and free of communicable disease.

There are certain advantages in keeping the experimental animals in small groups: it simplifies identification and stock taking, and the keeping of records; and allows frequent inspection for dead animals; it reduces the death rate resulting from fighting. The spread of infection is limited to one agent; *viz.*, the cleaning implements and hands of the caretaker.

Introduction of the Virus.—As already stated, when the study was begun Topley's papers had not appeared. His method of placing a known amount of culture on small bits of bread to be consumed by mice from which food had been withheld for 24 hours is efficacious

² Topley, W. W. C., *J. Hyg.*, 1920-21, xix, 350.

and more accurate than our first attempts by allowing hungry mice to drink milk from a tube. In order to give the exact dose to each mouse, we later introduced the bacteria suspended in milk directly into the stomach through a small silver tube. In this way the dose can be accurately measured and the use of more than 1 cage for each 5 mice obviated for the preliminary step of infecting the first mice.

Inspection.—The cages were examined three times daily except Sunday, when only one inspection was made. Even under these conditions it sometimes happens, especially on the first round of the day, that only a partially eaten body of a mouse is found. The survivors quickly attack the body of a dead mouse and devour the softer parts. Earlier in the experiments, when we had little experience, the number of deaths which could not be definitely determined as due to *Bacillus typhi murium* infection was proportionately larger than in the later series.

The bodies collected at 9 a.m. rounds were autopsied at 10 a.m. Those collected later were kept in the refrigerator (+4°C.) until the following morning.

Records.—In following the progress of the infection, two charts were kept: (a) plot of the total number of deaths for the whole series, according to days, and (b) spot map using colored pins showing the location of the mice which died and the number of days elapsed since the beginning of the exposure.

Autopsy Technique.—The notes at autopsy included the weight of the body and of the spleen, and a brief description of the macroscopic appearances of the spleen, intestines, liver, and gall bladder. The bacteriological examinations of the spleen, intestinal contents, and gall bladder were carried out in the following manner. A small bit of the material was transferred to a tube containing 5 cc. of brilliant green broth (brilliant green 1:200,000 in broth of pH 7.4) and crushed inside the tube. To the tube there was then added 0.25 cc. of a 1 per cent sterile aqueous solution of lead acetate. The tube was incubated at 37°C. over night. If on the following morning the precipitate in the tube was brown, showing evidence of sulfide production (presumptive test of the presence of *Bacillus typhi murium*), a loopful of the broth was smeared on lactose-saccharose-neutral red agar, pH 7.4, plates containing 1:400,000 brilliant green. Colonies from these

plates were fished into lead acetate agar tubes containing four sugars:³ lactose, raffinose, saccharose, and salicin, 0.25 per cent of each. Cultures having the characteristic reactions of *Bacillus typhi murium* were then agglutinated with two monotypical immune sera. The longer incubation period for brilliant green broth tubes has revealed a higher percentage of deaths attributable to mouse typhoid than was obtained with the usual procedure of 1 hour's incubation in a water bath.⁴ The addition of lead acetate to the broth greatly reduces the number of brilliant green plates required. The first few hundred examinations with this technique were controlled by the usual culture method resulting in a gain of about 10 per cent in the positive findings. The lead acetate did not fail in a single instance to reveal *Bacillus typhi murium*, and in 125 tubes, in which there was no browning controlled by plating, no *Bacillus typhi murium* was found. Many brilliant green tubes containing feces may show the sulfide reaction when no *Bacillus typhi murium* is present, due to the growth of other sulfide-producing organisms such as *Bacillus alkaligenes*, etc. The plating on brilliant green and transferring of the colonies to the four sugar tubes control this point.

The final agglutination test is highly important as endemic strains, differing antigenically from the strain used to induce the artificial infection, are sometimes found in mice succumbing in course of the epidemic experimentally set up.

³ The composition of this medium is as follows:

Beef extract.....	3 gm.
Peptone.....	10 "
NaCl.....	5 "
Agar.....	15 "
Lactose.....	0.25 per cent.
Raffinose.....	0.25 " "
Saccharose.....	0.25 " "
Salicin.....	0.25 " "
Andrade indicator.....	1 " "
Distilled water.....	1,000 cc.

pH 7.2

Lead acetate.....	{ 1 cc. of 0.25 per cent solution to each 4 cc. tube of medium.
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⁴ Undoubtedly in our earlier series many negative results were recorded which with the newer technique would have been found positive.

Bacillus typhi murium Employed.

A bacillus conforming to the cultural characteristics of *Bacillus typhi murium* and pathogenic for mice was isolated from an epizootic among a cancer breeding stock.¹ This strain (Mouse Typhoid I) was passed by intraperitoneal injection through 5 mice. A suspension containing 1:20 of an 18 hour culture on slant agar was then fed to a mouse, and on the death of this animal the bacillus recovered, after being identified culturally as belonging to the same group, was fed to 4 mice in succession. As it happened, later immunological tests showed the strain recovered from the final mouse of this series to be antigenically different from the original strain but having identical fermentative properties with those of so called *Bacillus typhi murium* and being pathogenic for mice. It is probable that this strain which we term Mouse Typhoid II was enzootic among the stock used in the preliminary experiments, replacing somewhere in the series of 4 mice the strain used originally to induce infection. It came out in later studies that the second large outbreak among the cancer breeding stock¹ was caused by a strain antigenically identical with this strain. However this may be, the strain acquired highly invasive powers, as the experiments to be described will show. From this point on, this strain appeared in the animals succumbing to the mouse typhoid arising in the course of our experiments except in a few instances in which an immunologically different strain was obtained in culture. The fact of this substitution of strains emphasizes the importance of making regular immunological tests on all strains employed in and recovered during the experiments.

In referring to this bacillus with which our experiments were conducted as *Bacillus typhi murium*, we would have it understood that it belongs to the class of bacilli embraced under this term and was later found to be indistinguishable by immunological tests from *Bacillus pestis caviæ* of the paratyphoid B group. The immunological reactions of the bacillus will be described in a separate communication.^{5,6}

Preliminary Culture Feeding.

Feeding Series B.—November 2, 1919. 18 normal mice, after fasting 24 hours, were fed during 24 hours with milk containing 1:20 culture of Mouse Typhoid II

⁵ Amoss, H. L., and Haselbauer, P. P., *J. Exp. Med.*, 1922, xxxvi, 107.

⁶ Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 97.

from an 18 hour growth on slant agar. During the next 18 days, 15 died and yielded positive cultures of the same bacillus in the feces and spleen; in 8 the bacillus was recovered also from the gall bladder. The deaths took place as follows: 3 occurred in 7 days; 4 in 8 days; 2 in 9 days; 2 in 10 days; 1 each in 11, 15, 17, and 18 days. On the 36th day the 3 surviving mice were killed, of which 2 proved fecal carriers. The bacillus was not found in the spleen or gall bladder.

Series C.—The day after the feeding of the culture in milk just described, 13 mice (Series C) were placed in 2 cages of 5 and 8 respectively, and placed beside the cages containing the fed animals. They were all cared for by the same attendant. Of the cage of 5 mice, 1 only succumbed to mouse typhoid and that on the 12th day. Of the other cage, all 8 succumbed, on the 11th, 13th, 14th, 15th, 17th (2), 19th, and 25th days respectively. The unequal distribution of deaths in this series need not be considered now; but the main point is that already the culture Mouse Typhoid II had exhibited power to induce fatal infection through mediate contact as well as through direct feeding. Moreover, the contact mice succumbed in periods not exceeding those of certain of the fed mice.

Series D.—13 days after the feeding experiment of November 2, 24 mice in 8 cages (Series D) were placed beside the cages containing the 11 surviving mice of Series C. 11 died and 8 yielded positive cultures. At the expiration of 60 days, the remaining 13 were killed. 5 of them proved fecal carriers.

Experimental Epidemic.

The culture Mouse Typhoid II was now regarded as probably capable of producing mouse typhoid by ingestion in a large proportion of the fed mice and also of inducing that disease in exposed or contact mice not directly fed. Whether it possessed also the particular qualities which might be required in order that the spread from animal to animal should take place in the manner common in epidemics remained to be ascertained. An experiment to test this point was next designed.

Series E.—December 12, 1919. Food was withheld for 24 hours from 10 mice. Each mouse was then given milk to drink containing a heavy suspension of culture Mouse Typhoid II. It is estimated that each animal received approximately 1:20 of an 18 hour agar slant culture. At the end of 24 hours the mice were transferred to clean cages, 5 in each. With this experiment in view, 100 normal mice were assembled on December 8, into 20 cages, and arranged in positions indicated by the following diagram.

5 cages of normal mice.	2 cages of mice fed with culture.	15 cages of normal mice.
□ □ □ □ □	□ □	□ □ □ □ □ □ □ □ □ □ □ □ □ □ □

These mice had been free of fatalities. Just here it should be stated that the mice assembled for all the experiments were not only home-bred and of a stock free of disease, but when introduced into the experiment they were of an average weight of 13 gm. and age of 4 weeks.

The series of events were now as follows: Of the 5 mice in feeding cage 1, 2 died on the 8th, 1 each on the 13th, 14th, and 15th days. From all, cultures of Mouse Typhoid II were obtained. Of the 5 mice in feeding cage 2, 1 each died on the 12th, 13th, and 17th days. The 2 remaining mice were killed on the 60th day; cultures were negative.

Of the 100 mice in the 20 contact cages, the 1st animal died on the 15th day and the next on the 17th day. Within 60 days a total of 10 mice had succumbed. Of the 90 survivors, 7 (or 8 per cent) proved fecal carriers. Only 11 of the 20 cages showed infected mice as indicated either by death or carriage. The position of the mice, in respect to the purposely infected animals and the direction of the feeding or the cleaning of the cages, had no appreciable effect on the incidence of the infection. Obviously the attendant, in spite of more than ordinary precautions to keep his hands clean, early became contaminated and spread the contamination unwittingly and irregularly after the 1st day or two of his operations.

It is evident that what was produced in this experiment (Series E) was not an epidemic outbreak but rather a sporadic occurrence of mouse typhoid. But the impending conditions seem to have been significant. For on January 15, 1920, 79 normal mice in 17 cages (Series F) were assembled and placed on racks immediately next to the cages of Series E on the 34th day after the beginning of that experiment. The effect was striking. No deaths occurred within the first 5 days, but there were 4 in the second 5 day period. The highest recorded were in the third 5 day period, and this wave quickly subsided. Two smaller waves appeared with their crests in the seventh and the ninth 5 day periods. The distribution of deaths in cages and periods is shown below.

Distribution of Deaths in Series F.

Days.	Deaths in cages.	Total No. of deaths in period.
1-4	0	0
5-9	Nos. 6, 13, 13, 16	4
10-14	" 1, 3, 4, 4, 5, 6, 7, 8, 9, 15	10
15-19	" 1, 2, 2, 4, 5, 6, 6, 10, 13	9
20-24	" 1, 4, 8, 11, 11	5
25-29	" 2, 3	2
30-34	" 2, 3, 11, 12, 15, 16, 17	7
35-39	" 2, 4, 13, 16	4
40-44	" 1, 8, 8, 8, 15	5
45-49	" 7, 13, 17	3
50-54	" 12, 14	2
55-65	" 13, 14	2

About 75 days after exposure the survivors of Series F, 24 in number, were killed and examined as carriers. 1 only was found. The following tabulation summarizes the results.

Total mortality.....	55, or	70 per cent.
Corrected "	41, "	52 " "
Cage attack rate.....	17, "	100 " "
Carriers among survivors.....	(1 in 24)	4 " "

A word of explanation is required regarding the terms total and corrected mortality. The latter refers to dead mice from which bacillus Mouse Typhoid II was recovered in cultures. The technique employed at this date was one in which the brilliant broth tubes were incubated for 1 hour before plating. Further studies showed that with this method many of these organisms are missed. This fact taken together with results obtained at a later date leads us to infer that the gross mortality figure as given is very nearly correct.

Thus it appears that once what may be termed sporadic cases of mouse typhoid are made to arise in a population previously free of this infection, the introduction of fresh, previously unexposed individuals may suffice, after a certain delayed or incubation period of 5 to 10 days, to bring about a sharp outbreak of cases which may reach epidemic proportions. In this particular instance every cage was attacked and the death rate very high.

Series G.—On February 13, 1920, or 29 days after the preceding or F series was started and at a time when the deaths among the latter had fallen, for the 5 days preceding, to 4, 48 normal gray tame mice of the average ages and sizes to the white mice employed up to this time, contained in 10 cages, were introduced into the mouse village and placed next the survivors of Series F. Instances of mouse typhoid arose among them and 25 mice died. On April 12, or 2 months after the placing of these mice in the village, the survivors were killed and examined for carriage of the bacillus Mouse Typhoid II. The following tabulation summarizes the results of this test.

Total mortality.....	25, or	52 per cent.
Corrected "	24, "	50 " "
Cage attack rate	10, "	100 " "
Carriers among survivors.....	(4 in 23)	17 " "

In brief, this series behaved very much as did the preceding one in respect to the several points covered in the study. It may be desirable

to point out the close correspondence between the total and corrected mortalities resulting from the improved bacteriological technique described on page 27.

It now became evident that it was possible to inaugurate epidemics of mouse typhoid among a healthy previously unexposed mouse population. Up to this time the events described are remarkably consistent. But as the experiment was continued by the successive introduction of fresh, vulnerable material, variations (or better stated perhaps wider fluctuations) made themselves apparent as we will now proceed to show.

At this point one or two incidental observations are called for. In the mouse room as arranged the racks carrying the cages were placed along the sides of two opposite walls, so that the cages could be made contiguous or could be separated by the distance of the width (12 feet) of the room. This separation did not in itself affect the closeness of contact, since in no instance did the mice actually mingle, but, as already stated, the intermediation of infected and non-infected animals was secured through the hands of the attendant.

However, the separation of cages by the width of the room made possible the carrying out of devices which might affect the mediation. For example, the attendant was made to use approved methods of hand sterilization⁷ and to clean the cages and feed the mice on the side of the room away from the series in which the infection existed, before tending the latter.

Series H.—Thus on February 13, 1920, or the same day that Series G was brought into the village and placed in cages immediately adjoining those of Series F, 100 healthy mice were assembled in 20 cages (Series H) and placed by themselves on shelves along the opposite wall at a distance from those already in the room, where they were kept for 2 weeks. As deaths among these mice began within 2 weeks, it is evident that the carrying out of sterilization by the attendant did not suffice to render his hands free of contamination with the strain of bacillus employed in the infection experiments. The series was now brought over and placed next to Series I, described below. Deaths among them continued so that at the end of 2 months, April 12, the results were as follows:

⁷ The cleaning of the hands of the attendant was carried out in the following order. 10 minutes scrubbing with hand brush, using tincture of green soap. Wash in warm tap water. 2 minutes in potassium permanganate solution. Then immerse in saturated solution of oxalic acid until stain is removed (3 to 5 minutes). Rinse in tap water.

Total mortality.....	33 per cent.
Corrected "	25 " "
Cage attack rate.....	(14 in 20) 70 " "
Carriers among survivors.....	(8 " 67) 12 " "

The lowered death rate and the reduced cage attack rate are at once apparent. That the events summarized in the tabulation accompanying Series H are not mere exceptions is shown by the next experiment.

Series I.—February 18, 1920. 177 healthy mice, distributed in 36 cages, were brought into the village and placed adjoining and in line with Series G. These healthy animals also were tended without sterilization of hands and tools, after Series G, in which the infection was still proceeding. The detached Series H was cared for first of all. It was supposed that should the infection appear among the mice of Series I, it would attain a degree of activity capable of being accelerated by the available mass of new infectible material introduced at about the period of the preliminary outburst, while the mice in the detached Series H might acquire some resistance due to slow infiltration of virus. As a matter of fact, the two series, H and I, behaved quite as if they had been one series and all the mice had been brought into the village at one time. The experiment involving Series I was terminated on May 18, or after 3 months. The tabulation gives the final outcome.

Total mortality.....	64, or 36 per cent.
Corrected "	47, " 26 " "
Cage attack rate.....	(28 in 36) 80 " "
Carriers among survivors	(14 " 113) 12 " "

Review.

It is not our intention to enter into a minute discussion in this place of the significance of these experimental data. Such discussion as we purpose to give the subject will be presented in connection with the next paper in which the succession of events taking place day by day in the several series will be described.⁸ We prefer merely to pass in review in this place the salient facts connected with the experiments detailed.

The feeding of the mice exposed in the village and the cleaning of the cages were done by one person and always in the same direction.

⁸ Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 45.

As each new series was introduced, its cages were first tended. Series G and I were brought into the mouse village when the epidemic in Series F was at the crest of the wave. Series F (79 mice) was assembled and exposed on January 15, Series G (48 mice) on February 13, Series H (100 mice at distance until February 27) on February 13, Series I (177 mice) on February 18. Series F, G, and I were contiguous; Series H was separated by the distance (width) of the room (12 feet). Before the cages of Series H were touched, the attendant scrubbed his hands and nails with soap and brush for 10 minutes and used permanganate of potash and oxalic acid as disinfecting solutions. After 2 weeks separation Series H was brought into contact with Series I.

The several series may be regarded as having been placed in a single line, with the cleaning and feeding carried out in one direction. Thus following Series F there are 10 cages of Series G containing 5 mice each, followed by 36 cages of Series I and 20 cages of Series H, also of 5 mice each. Numbering the cages in order, Series G would be covered by cages 1 to 10; Series I by cages 11 to 46; and Series H by cages 47 to 66 inclusive. It might be expected that mice would be first attacked and die in cages 1 to 5 and that the infection would spread by contiguity. But the order is not so regular as this and the contrary may happen, for the first mice to die of mouse typhoid were in cage 7; then in cages 2, 3, and 4; then in Nos. 2, 3, and 5; then in Nos. 9 and 10. In Series I, of 36 cages, exposed 5 days after Series G, the distribution was as follows:

Days.	Deaths in cages.
1-4	Nos. 20, 29
5-9	" 11, 16, 17, 23, 25, 34
10-14	" 18, 24, 27, 28, 33, 34, 39
15-19	" 11, 13, 14
20-24	" 13, 23, 30, 32, 34
25-29	" 13, 26, 32, 33
30-34	No. 30
35-39	Nos. 26, 27
40-44	" 24, 29
45-59	" 11, 19, 21, 24

The one safe deduction from this tabulation seems to be that a wide but not uniform distribution of the bacillus is quickly brought about by the attendant through which individual mice, in an entirely unpredictable order, take it up and fall victims to the infection ensuing. Once this has happened the cages must soon become contaminated widely by the excrement carrying the bacillus and all the remaining mice should receive the organism. The sources of the subsequent irregular events can only be inferred as depending upon such factors as dosage and possibly fluctuations in pathogenic activity of the bacillus, and upon variations in the resisting powers of the mice. These factors are the ones commonly invoked to explain such vagaries of case incidence of the communicable diseases as here presented and are set down here not as finalities or even as matters for discussion, but rather to emphasize a parallelism existing between the natural, so called, infections in man and animals and those purposely set up, as in the instance being considered, which may be used in putting the next question to be answered by experiment.

In concluding this presentation, a table has been prepared of the five series of mice exposed to infection with the bacillus of mouse typhoid (Table I). The number of mice in each series varied from 48 to 177. In the table, Series I, which contained the largest number given, is broken up into three parts called first, middle, and last, according to the degree of removal of the cages at the time they were brought into the mouse village from the mice which were already potentially infected. It is not suggested that the factor of nearness or remoteness is a controlling one, but the division is interesting as bringing out again the element in the process of distribution of the infection which for the present is merely termed vagary. It is to be kept in mind that the quality of mice in the three divisions was superficially homogeneous. The only mice fed with culture were 10 of Series E. The rest of the animals received the bacillus through the exigencies of contact between them, their habitation, and the hands of the attendant.

The figures in Table I not only speak for themselves but have already been discussed in the earlier pages of this paper. The exception is Series I in which the three groups of approximately 60 mice each, selected by degree of removal from the older infected series into

relation with which they were brought, show a mortality of 38, 56, and 15 per cent respectively. Since the first group of 60, immediately next the older and already infected series, gave a lower mortality than the middle and further removed group, the very low mortality in the last group can hardly be accounted for by position alone.

TABLE I.
Composite Table of the Five Series.

Series.	No. of mice in series.	Mortality.	Corrected mortality.	Carrier rate among survivors.	Carrier rate in total No.	Cage attack rate.
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
E*.....	100	10	10	8	7	55
F.....	85	70	52	4	1	100
G.....	48	52	50	17	8	100
(First.....	60	38	22	11	7	83
I Middle.....	60	56	52	4	2	100
(Last.....	57	15	5	18	15	50
H.....	100	33	25	15	10	70

* The infection in this series was produced by feeding with a suspension of *B. typhi murium* 10 of the mice.

The tabulation brings out a striking irregularity between carrier and death rate. Thus of 14 carriers, 8 were found in the same number (8) of cages in which no deaths from mouse typhoid took place, while 6 were found in 29 cages in which deaths from the infection took place. The distribution of carriers according to deaths in the cages is as follows:

No. of deaths in cage.	Instances of carriage of bacillus.
0	8
1	3
2	1
3	2
4	0

Experiments with Strains Arising from a Single Bacillus.

Six strains were obtained from single cells of Mouse Typhoid II by Barber's method. In order to be certain that only single cells

were picked, the strains obtained were plated, a colony was picked, and from the 8 hour growth in broth a single cell was again isolated and the same process repeated. Thus before a strain was considered as arising from a single cell, the culture was analyzed by the Barber method three times.

Six such pure-line strains were obtained, three of which showed slightly greater virulence by intraperitoneal injection into mice, as shown in Table II.

TABLE II.

Results of Intraperitoneal Injection of 1:30,000 of a Culture of Each of Six Single Cell Strains of Mouse Typhoid II.*

Weight of mice.	Length of life.					
	Strain.					
	A	D	E	F	G	H
gm.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.
13	52	44	66	20	66	64
	60	60	78	54	72	66
14	75	78	100	64	84	72
	88	78	105	66	90	78
15	80	82	115	72	94	100
	90	86	134	78	134	138
16	96	94	150	84	150	200
	111	110	160	99	200	255
17	130	130	255	115	408	260
	140	132	S.†	200	520	495

* The suspensions were standardized in a turbidimeter.

† S. indicates survived.

Comparison of the Virulence of Single Strain A with the Composite or Original Strain of Mouse Typhoid II.—Since the results of intraperitoneal injections (virulence tests) of mice with the mouse typhoid organisms vary considerably, it becomes necessary to use large numbers of mice in each experiment. With the view of determining what mice grouped according to weight would yield the most concordant results, the following experiment was made.

Experiment 1.—Mice were selected from a large stock until 10 of each weight from 10 to 17 gm. were obtained. Three lots of 6 each, composed of individuals weighing 18, 19, and 20, and 1 weighing 21 gm., were selected. Each of the 99 mice received intraperitoneally in 1 cc. of salt solution 1:30,000 of a 16 hour slant agar culture of Mouse Typhoid II. This strain had been plated repeatedly so that the culture represents the descendants of a single colony.

The number of hours before death is recorded for each mouse in Table III.

TABLE III.

Relation of Weight to Susceptibility to Intraperitoneal Injection of Mouse Typhoid, Strain II.

November 30, 1920. Each mouse received intraperitoneally in 1 cc. 1:30,000 of a 16 hour growth on pH 7.4 agar; suspension standardized in a turbidimeter.

Length of life.											
Weight of mice.											
10-10.5 gm.	11-11.5 gm.	12-12.5 gm.	13-13.5 gm.	14-14.5 gm.	15-15.5 gm.	16-16.5 gm.	17-17.5 gm.	18-18.5 gm.	19-19.5 gm.	20-20.5 gm.	21-21.5 gm.
No. of mice.											
10	10	10	10	10	10	10	10	6	6	6	1
hrs.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.
20	20	20	20	49	20	20	20	46	20	20	89
20	20	27	25	66	30	59	20	73	60	20	
25	20	28	40	72	52	64	20	86	70	30	
48	41	60	50	75	52	86	27	106	75	60	
50	63	60	65	78	60	89	30	115	91	67	
69	67	72	74	79	73	90	34	130	96	75	
86	67	83	75	86*	90	118	75				
98	67	86	89	115	115	118	86				
106	86	93	93	125	130	118	88				
110	113	104	106	160	144	160	90				

The number of deaths recorded in each group at 50, 75, 100, and 150 hours shows little correspondence, so that no conclusion can be reached in the matter of the selection of the most suitable group for testing virulence. If, however, a single cell strain of this micro-organism is employed, there appears in the same kind of experiment a definite relation between the susceptibility and age groups, as is shown in Experiment 2.

Experiment 2.—120 mice were selected so that there were groups of 10 of each weight from 10 to 21 gm. Such mice received intraperitoneally in 1 cc. 1:30,000 of a 16 hour slant agar growth of single cell strain Mouse Typhoid II A. The suspensions were standardized in a turbidimeter to the density of the suspension used in Experiment 1. The results are shown in Table IV.

TABLE IV.

Relation of Weight to Susceptibility to Intraperitoneal Injection of Single Cell Strain Mouse Typhoid II A.

January 13, 1921. Each mouse received intraperitoneally in 1 cc. 1:30,000 of a 16 hour growth on pH 7.4 agar; suspension standardized in a turbidimeter.

Length of life.											
Weight of mice.											
10-10.5 gm.	11-11.5 gm.	12-12.5 gm.	13-13.5 gm.	14-14.5 gm.	15-15.5 gm.	16-16.5 gm.	17-17.5 gm.	18-18.5 gm.	19-19.5 gm.	20-20.5 gm.	21-21.5 gm.
No. of mice.											
10	10	10	10	10	10	10	10	10	10	10	10
hrs.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.
50	20	26	14	20	20	40	70	16	18	96	90
58	20	28	16	60	58	58	86	75	90	124	117
64	22	58	52	67	60	64	88	90	96	136	168
66	26	68	70	70	74	70	90	106	132	150	168
70	34	70	74	85	75	80	111	117	134	188	186
74	42	74	106	100	88	90	132	142	158	202	206
85	52	90	114	132	148	124	130	184	266	248	208
90	100	96	122	132	168	140	140	230	304	260	260
154	108	108	140	150	254	312	250	264	308	296	404
158	115	115	180	163	256	S.	266	S.	624	404	630

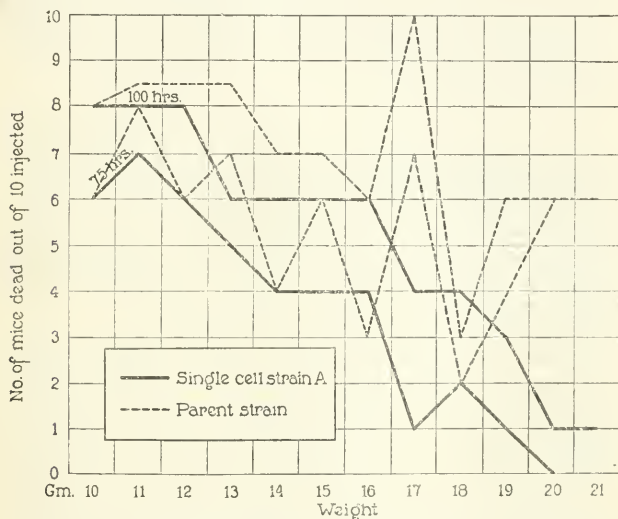
A comparison of the isolethal lines at 75 and 100 hours in Experiments 1 and 2 is shown graphically in Text-Fig. 1.

Of the two, the parent strain seems to be more virulent. The irregularity of the results in Experiment 1 and the obvious correlation between body weight and susceptibility shown in Experiment 2 with the single cell strain point to individual variation among the micro-organisms comprising the parent strain.

Pathogenicity of Single Cell Strains Administered by Mouth. Experiment 3.—The power to infect by mouth of three of the single cell strains was tested by allowing groups of 8 mice each to drink milk containing living cultures of the strain to be

tested. Of 8 mice receiving Strain A, 5 died and 3 were living after 30 days; with Strain F 6 mice died and with Strain H only 2 died. Strain F was therefore selected in the experiment to be described, in which attempts were made to start an epidemic of mouse typhoid under the conditions known to be favorable.

Series O. Experiment 4.—February 10, 1921. Each of 10 normal mice from which food had been withheld for 24 hours was fed by stomach tube 1:250 of a 16 hour slant agar growth of pure-line Strain F.



TEXT-FIG. 1. Comparison of the relation of the virulence of a single cell strain and of the parent strain to body weight of mice. The injections were made intraperitoneally. The difference in weight of the 10 mice in a given group was not more than 0.5 gm.

February 11. The 10 mice in 2 cages, 5 in each, were placed midway on a shelf among 20 other cages, each containing 5 normal mice. During the following 46 days, 6 of the feeders and 7 of the contacts died of mouse typhoid.

On the 46th day of observation 100 normal mice in 20 cages (Series P) were brought into line with Series O. There was no sharp increase in deaths among the normal mice added or among the original. Thus among the new mice (Series P) the first death from mouse typhoid occurred 3 days later, and one on the 14th,

18th, and 36th days. Within 98 days only 11 of the mice died. During this period 10 of Series O died of mouse typhoid, making a total of 17 of the contacts in Series O and 6 of the feeders.

Since the addition of fresh normal mice in the above experiment with pure-line Strain F did not result in an outbreak of mouse typhoid under conditions which in our experience constantly incite epidemics with Mouse Typhoid II, another pure-line strain was employed in the next experiment under slightly different conditions; *viz.*, the new mice were added on the 31st day as in Series K, L, and M⁹ instead of the 46th day as in Series O and P.

Series Q. Experiment 5.—June 8, 1921. Each of 10 normal mice from which food had been withheld for 24 hours was allowed to drink milk containing 1:20 of a 16 hour agar slant growth of pure-line Strain A, isolated from Mouse Typhoid II and belonging to the three more virulent pure-line strains.

On the following day the mice were placed in 2 cages, 5 in each, situated midway in a line of 20 cages, each containing 5 normal mice. During the following 30 days only 2 of the contact mice died. None of the feeders succumbed. On the 31st day, 100 normal mice in 20 cages (Series R) were added in line with Series Q. During the next 112 days only 7 of these mice died.

Comment.—Pure-line Strains F and A came from single cells picked from Mouse Typhoid II, the strain which was used to start the long replacement series, K, L, and M. It will be seen in another paper in this issue⁸ that in the latter series the addition of new normal mice during a quiescent period was followed shortly by a new outbreak, first among the new or added mice and then among the old mice; that is, mice which had been for some time exposed to the virus. Under these same conditions of feeding, arrangement, and time under which the epidemic waves in Series K, L, and M were established with Mouse Typhoid II, the pure-line strains signally failed to induce an epidemic. Thus the individuals from which Strains F and A descended lacked the power to produce an epidemic under the same conditions which sufficed for the composite strain Mouse Typhoid II to incite an epidemic outbreak.

⁹ These series are described at length on p. 46 of this issue.

SUMMARY.

In this paper we have described the first part of an experimental study of the epidemiology of mouse typhoid. One set of data has been presented on the basis of which little or no analysis has been attempted. The immediate object has been rather to collect materials than to undertake to account for the phenomena encountered. It is obvious that the factors involved in the inquiry are intricate, but it is believed that they are not necessarily or all beyond disentanglement. About 500 mice in all have been studied in an experimental village, brought together in increments among a population in which mouse typhoid experimentally induced was prevailing.

The results have been presented according to two phenomena; namely, mortality or death rate, and bacillus carriage rate. The material does not lend itself to consideration according to morbidity rates. If it were established that every instance of attack, when not fatal, was attended by carrier production for the bacillus of mouse typhoid, reliable morbidity tables could be constructed. In the absence of this certain criterion, the materials here presented can be dealt with only as mortality data. This fact is attended with obvious disadvantages in respect to the epidemiological material assembled regarding infectious disease in man. In spite, however, of the drawbacks, it is already evident that the results obtained by the sort of inquiry here described may come to throw no inconsiderable light on moot problems on the origin, mode of spread, and manner of decline of epidemic diseases in general.

The analysis of the strains by selecting single cells and thus establishing substrains has yielded results which may eventually have value in explaining fluctuations in virulence. Among the positive data arising from the experiments with such cultures are, first, that there have been obtained by mechanical means from the ordinary bacteriologically pure culture, single cell strains exhibiting slightly different pathogenic activity, whether administered by mouth or parenterally, and second, that more regular results are obtained with intraperitoneal injections of these strains than with the parent strain. Among the negative results to be recorded are the failures of two single cell strains to incite an epidemic among mice under conditions known to be suitable when the parent strain is used.

EXPERIMENTAL EPIDEMIOLOGY.

II. EFFECT OF THE ADDITION OF HEALTHY MICE TO A POPULATION SUFFERING FROM MOUSE TYPHOID.

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In earlier papers of this series an ordinary or spontaneous epidemic¹ of mouse typhoid and an artificially induced outbreak² of the same disease have been described. The purpose of the present paper³ is to consider more minutely the effect of bringing a healthy stock of mice into a community in which mouse typhoid is prevailing. The essential fact that such an addition of healthy mice leads to the transfer of the infection from the old population to the new comers, and the gross mortality figures thus resulting² have been pointed out. But the manner of the spread of the disease from the old to the new, and the reaction or effect of the revived epidemic on the old and previously surviving individuals, need to be dealt with in detail.

Method.

Three series of 100 mice each were assembled separately. The first, Series K, was used to start the outbreak by placing in it 10 mice which had been fed with living cultures, while the other two, Series L and M, were isolated to be brought 30 days later into contiguity with the first. After the three series had been brought together the infection spread, resulting in an outbreak which then subsided to a low ebb in which no deaths occurred for 1 week. Other new healthy mice were then introduced to bring the total number up to the original strength.

¹ Lynch, C. J., *J. Exp. Med.*, 1922, xxxvi, 15.

² Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 25.

³ An abstract of this paper was presented at the 36th Session of the Association of American Physicians, Atlantic City, May, 1921 (Flexner, S., and Amoss, H. L., *Tr. Assn. Am. Phys.*, 1921, xxxvi, 34).

After the new wave thus excited had receded, it became the rule to recruit again, by the introduction of healthy mice. Twelve such replacements were made during the period covered by this report.

EXPERIMENTAL.

Exciting the Epidemic.

Experiment 1.—Series K: May 1, 1920. 100 healthy mice were assembled in 20 cages of 5 each. May 3. 1 mouse found dead; no *B. typhi murium* detected. May 13. Second mouse found dead and *B. typhi murium* isolated from feces.⁴ May 20. As no further deaths occurred, there were placed, midway in the series, 2 cages of 5 mice each, which had been fed during 24 hours on milk containing a suspension of *B. typhi murium*. It is estimated that each of the 10 mice received about 1:20 of an 18 hour agar slant culture. During the next 23 days 12 deaths occurred from mouse typhoid, 6 among the 10 fed and 6 among the 100 contact mice. The last of these deaths occurred on June 21, on which date Series L and M were brought into the room containing the infected Series K.

Series L: 100 mice had been assembled also on May 1. Between this date and June 21, 3 of the mice had died, and *B. typhi murium*⁵ had been recovered from the spleen and feces in each instance.⁴ On June 21 the 20 cages of Series L were placed in a row immediately adjoining those of Series K.

Series M: This series,⁶ numbering 100 mice, had also been assembled on May 1. Between that date and June 21, when the 20 cages were brought into the room with infected Series K and placed immediately next the cages of Series L, 3 mice had died, and *B. typhi murium*⁵ had been obtained from the spleen and feces of each.⁴

It is shown in Text-fig. 1 that the fatalities in Series K resulting from contact with the fed mice were very low—6 per cent. With-

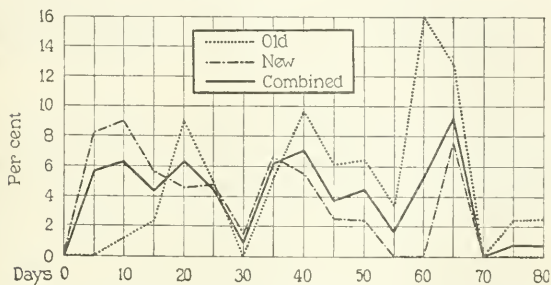
⁴ Cultures from the spleen and gall bladder were negative. Three deaths from mouse typhoid occurred in each of the other two series, L and M, before exposure to the experimental infection. All came from the same stock of about 3,000 mice among which there occurred 6 deaths from mouse typhoid during the spring of 1920. It is presumed that in assembling the series one or more carriers was included. However, the series were for this reason kept isolated for 20 to 30 days before the experiment was begun.

⁵ At this time the differences in Mouse Typhoid I and Mouse Typhoid II were not known.

⁶ Series L and M were identical. The former was contiguous with Series K and the latter 20 cages removed from Series K, but contiguous with Series L. These two series are kept separate in order to observe the effect of distance from the source of infection on the manner of spread, and also for convenience in keeping records.

in 5 days after Series L and M were brought in, 6 deaths occurred in Series L and 9 in Series M, a total of 15; and in the next 5 day period a total of 19 deaths occurred in the two series. No deaths occurred in Series K during the first 5 day period and only 1 death in the second 5 day period.

The immediate result, then, of the introduction of normal animals was an outbreak among those mice brought in, whereas deaths in Series K did not increase until during the third 5 day period. The epidemic progressed in a succession of waves in each of the three series. In the like Series L and M, the waves were synchronous throughout the period, and the crests were lower and the intervals between greater



TEXT-FIG. 1. Death rate by 5 day periods in Series K, combined death rate for Series L and M, and combined rate for the three series, during the first epidemic period.

as the epidemic proceeded. The wave in Series K lagged behind Series L and M, and in contrast rose higher and the intervals between them became shorter. The lag of Series K decreased until at the twelfth period the wave of this series was coincident with the smaller Series L and M waves.

The three series were kept intact and without increments for 70 days after the normal mice had been brought into contact with Series K, at which time the epidemic had reached a low ebb. The total mortality among the approximately 300 mice in the entire period of 85 days after the introduction of the new mice was 54 per cent; in Series K 62 per cent (56 out of 91); in Series L 40 per cent (38 out of 97); and in Series M 61 per cent (59 out of 97).

The mortality among the added Series L and M combined was 50 per cent, while for the same period, the mortality in Series K (exposed from the first to the culture-fed group) was 62 per cent.

Comment.

The conditions of the experiment involving Series K, L, and M were similar to those described in the first paper of this series, in which first a sporadic outbreak of mouse typhoid was started by bringing a small number of mice purposely fed with a culture of *Bacillus typhi murium* into an assemblage of healthy mice; and the sporadic incidence was later fanned into an epidemic outbreak by the introduction of fresh groups of healthy mice at a time at which the occasional deaths had practically ceased. Hence the events recorded in the former paper seem not to have been accidental in nature but rather to follow a certain rule.

The course of the fatalities in the K, L, and M series is a definite one. In the first place the distribution of deaths in the cages was such as to point, just as in the earlier experiments, to an early general dissemination of the bacillus. Thus the first fatalities occurred in cages remote from those of the fed mice. But more significant is the fact that once the infection appeared among the mice of the L and M series, the number of fatalities not only rose steadily to a maximum, but was reflected backward to the K series in which no further deaths were taking place at the time. The precise manner of the rise in the L and M series, the exact period of delay in the K series induced by the occurrences in the former series, and the final result, or sum total of fatalities in each series, are shown graphically in Text-fig. 1.

To undertake to account for these happenings is to enter, at the moment, the realm of speculation. Two possible explanations present themselves. According to one it may be supposed that the bacillus of mouse typhoid was whipped into an exalted virulence by being rapidly passed from one susceptible mouse to another until this was so great as not only to reach a degree of infectivity greatly augmented for the new mice of Series L and M, but also to increase its striking power for the mice of Series K which, for a 30 day period, had successfully weathered exposure to the purposely fed mice introduced into the midst of that group.

According to the other supposition the effects described do not depend primarily on augmentation of pathogenicity but rather on increase in mass of the bacillus and result not from virulence so much, or alone, as from dosage. This view involves the conception that favorable conditions of growth and multiplication of the bacilli in the exposed mice are the more decisive events. It would seem as if these favoring circumstances did not exist in the first series employed but did arise subsequently among the new exposures. Once, however, they were secured, the growth and multiplication became such as to overwhelm not only the new series but also the older, previously exposed, survivors, among which the death rate was finally greater than among the new.

Probably the precise differences in the percentage mortalities are not significant. Series L and M were practically homogeneous and yet the one (L) gave a mortality of 40, and the other (M) of 61 per cent, the latter agreeing almost exactly with that of the K series. Moreover, according to this comparison, there are no grounds for supposing that the survivors of Series K by merely having been previously exposed were better able to withstand the violence of a real epidemic than were the previously unexposed mice of Series L and M.

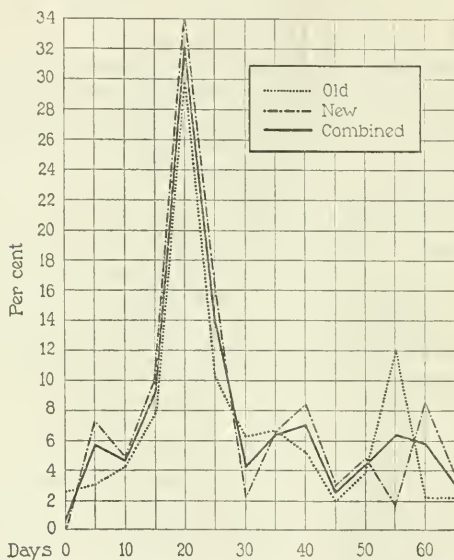
Hence it may be that the two factors mentioned, namely virulence and mass, are not only of high importance in themselves but also subject to such a degree of accurate measurement as to determine the potentially effective degree of each. All these considerations leave out of immediate account variation in the host—a factor at present not to be dealt with.

Effect of Replacements.

First Replacement, or R 1. Experiment 2.—September 9, 1920. The survivors of Series K, L, and M were observed during a period of 115 days at the expiration of which deaths took place seldom; during the final period of 7 days, no deaths at all occurred. At this juncture new mice from a healthy stock⁷ were added to each cage in numbers replacing the deaths, so that each cage again contained 5 mice and the total population in the room was recruited to 300. In order to

⁷ Among the stock of about 3,000 mice from which these mice were taken, there occurred 5 deaths from mouse typhoid in 6 months.

prevent losses from fighting between the old and the new mice, the latter were separated from the former by placing a coarse wire screen diagonally in the cage, separating it roughly into two compartments. While the mice themselves were thus grossly kept apart, yet they and their excrement came into contact.



TEXT-FIG. 2. Death rate by 5 day periods among the old and new mice, and combined rate, during the first replacement period.

The constitution of the several series as recruited was as follows: Series K now consisted of 98 mice,⁸ of which 38 were old and 60 new; Series L of 100, of which 57 were old and 43 new; Series M of 100, of which 27 were old and 73 new. Hence the relation of old or exposed to new or unexposed mice totalled 122 : 176.

The new events came quickly. Within 5 days 17 deaths occurred, 13 among the new and 4 among the old mice. In the next 5 days

⁸ This series was recruited to a total of 100 mice but 2 were killed in fighting.

there were 13 deaths, 8 among the new and 5 among the old. In the third 5 day period the deaths numbered 25, 16 of which were among the new and 9 among the old animals; while in the fourth 5 day period (20 days after the admixture of old and new mice) 79 deaths occurred, 48 of which were of new and 31 of old. This constituted the peak of the epidemic, after which the death rate fell off sharply.

These data are presented graphically in Text-fig. 2, from which it will be observed that the outbreak proceeded in two quite distinct waves among the new mice and in a steadily increasing progression among the old mice. This replacement, afterwards referred to as R 1, was observed for 65 days, during which a gross mortality from mouse typhoid of 69 per cent, made up of mortality K 86 per cent, L 68 per cent, and M 52 per cent, was noted.

Agglutinins in the Serum of the Survivors.

The serum of 56 of the survivors of the first replacement series was tested⁹ for agglutinin with Mouse Typhoid II in dilution from 1:20 to 1:160. The serum from 37, or 66 per cent, partially agglutinated the strain in a dilution of 1:40, and of these, 20 showed complete agglutination in 1:40 and 2 showed complete agglutination in 1:160. The serum of 27 of the 56 mice showed no agglutinin in 1:20. Further analysis of the results shows that the serum of the 20 mice agglutinated the strain completely in a dilution of 1:40,

⁹ The blood was collected from the mice by bleeding from the end of the tail. The mouse to be bled was placed in a small mailing case, one end of which was perforated and a single hole made in the other cap for the protrusion of the tail. The case containing the mouse was held in a clamp over a microscope lamp, the heat from which caused increased blood flow to the tail vessels. The tail was wiped with gauze moistened with 70 per cent alcohol, and after snipping with sharp scissors the drops of blood were allowed to flow by capillarity into a small bore, 1 cc. pipette graduated in hundredths. When 0.05 cc. had been collected, the end of the pipette was placed in isotonic salt solution and suction applied with a syringe until the mixture attained a total volume of 0.5 cc. The mixture now containing 10 per cent blood was delivered immediately into a small agglutination tube, mixed thoroughly, and allowed to clot. The following morning the clear fluid containing serum 1:20 was pipetted off with a small bore pipette, a syringe being used for suction.

while in 36 agglutination was either partial in 1:40 or absent. Of the former only 1 mouse, and of the latter 8 mice died during the second replacement series. Thus the death rate among the mice giving slight or no evidence of blood agglutinins was four times that noted among those possessing blood agglutinins.

Urinary Carriers.

Cultures of the urine of the same 56 mice were made on five occasions from November 15, 1920, to January 17, 1921. 35, or 62 per cent, were positive one or more times; of these 6 were positive during the entire period of 63 days. The serum of 5 of the 6 persistent urinary carriers agglutinated Mouse Typhoid II.

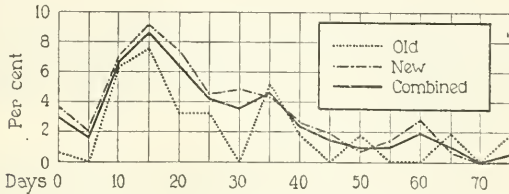
The results of the urine cultures and agglutination tests on the serum of the 56 mice are shown in Table I.

TABLE I.
Urine Cultures and Serum Agglutinins in 56 Survivors of the First Replacement Series.

	No.	Per cent.
Both agglutination and urine cultures positive	25	45
Agglutination positive, " " negative	12	21
No agglutination, urine cultures positive	10	18
" " " " negative	6	11

With these data before us we may now view the relative responses of the old or previously exposed and the new or not previously exposed mice to the impending infection. The number of old survivors which had already passed through an epidemic enduring 85 days and of which the total mortality was 58 per cent was 122. The death rate among these surviving animals induced by the new epidemic was 66 per cent, while the death rate among the 178 new or previously unexposed mice was 71 per cent. The percentage difference is too small to be significant, hence in all respects but one, namely that the new mice began to succumb before the first of the old, the two groups behaved in an identical manner. It is obvious that in the course of the second epidemic wave the gross mortality among the older mice exceeded that of the first epidemic, the proportions being as 58:71.

Second Replacement, or R 2. Experiment 3.—November 9, 1920. When the effects of the first replacement had subsided and no deaths at all occurred in a 5 day period, new mice, previously unexposed, were brought in as in the first replacement, to recruit the survivors in the three series to a total strength of 300. This second replacement was made 60 days after the first. The actual number of new mice introduced was 228, distributed as follows: Series K 87, Series L 82, Series M 59. Hence the proportion of old survivors to new population was as 72:228.



TEXT-FIG. 3. Death rate by 5 day periods among the old and new mice, and combined rate, during the second replacement period.

Text-fig. 3 shows that the new or R 2 mice began to die within 5 days, and 5 days later, or 10 days after the beginning of the experiment, deaths occurred among the survivors of the preceding epidemic. The experiment was allowed to run 75 days, at the expiration of which a total of 113 mice, or 38 per cent, had succumbed. The mortality in Series K was 29, L 40, and M 44 per cent, and among the old and the new mice it was 37.5 and 37.7 per cent, or virtual identity. That the old mice possessed no advantages over the new is further indicated by the patent fact that the number of the new (228) was approximately three times that of the old (72), thus increasing the probability of higher attack rate among the former. Ultimately the factors of age and size of the host may call for consideration. In those respects the surviving old differed materially from the younger and smaller new animals.

Third Replacement, or R 3. Experiment 4.—January 20, 1921. The third replacement of 114 new mice to recruit the new series up to 300 was made 75 days after the second. The series additions were almost equal: 66 in K, 65 in L, and 55 in M. During the next 60 days the total mortality was 14.3 per cent, distributed as regards old and new mice in proportion of 14 (old) to 14.9 (new) per cent.

This experiment was permitted to continue without interruption or modification for a period of 95 days, and it is worth while following the particular events which transpired. But first a few words are needed about the nature of the population composing the experiment. As is evident, the population is made up of mice of very different grades of exposure to mouse typhoid, since there have accumulated within it all the survivors of previous epidemics extending from May 20, 1920, of the first lot exposed, to January 20, 1921, when the third replacement was made. As Table II shows, the number of survivors of each replacement grows progressively smaller with each succeeding epidemic. Thus at the time of the third replacement period, what may be termed the combustible material consisted largely

TABLE II.

Distribution of Deaths According to Period of Introduction during the First 60 Days of the Third Replacement Period.

	Period of introduction of mice.			
	First epidemic (R).	First replacement (R 1).	Second replacement (R 2).	Third replacement (R 3).
No. of mice present.....	14	30	142	114
Deaths during first 60 days of Period R 3....	1	2	21	17
Death rate, <i>per cent.</i>	7	7	15	15
Survivors at 60th day of Period R 3.....	13	28	121	97

of the mice of the R 2 period. At the beginning of the R 3 period there were 14 mice remaining from the R period (first epidemic period), 30 from the R 1 period (first replacement), and 142 from the R 2 period. Reference to Table I shows that the mortality rate among the mice remaining from the R 2 period and the new mice was the same (15 per cent in each instance) and twice the rate observed (7 per cent) among the mice remaining from the R and R 1 periods.

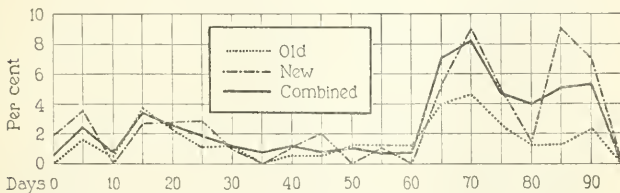
The events referred to may be summarized as follows: From the 25th to the 60th day, only an occasional death occurred in any of the series; for example, in Series K no death for 18 days; in Series L none for 29 days; in Series M none for 10 days. But at about the 60th day the deaths had reached epidemic proportions. This wave,

as it may be termed, was not induced intentionally but arose in the manner of similar waves in so called spontaneous epidemic outbreaks. Its duration was about 30 days, during which 73 (28 per cent) of 259 surviving mice after the first effect of the replacement subsided had succumbed. The distribution of deaths during this time, according to the period at which the mice were introduced, is given

TABLE III.

Distribution of Deaths According to Period of Introduction from the 60th to the 96th Days of the Third Replacement Period.

	Period of introduction of mice.			
	First epidemic (R.).	First replacement (R 1).	Second replacement (R 2).	Third replacement (R 3).
No. of mice present at 60th day.....	13	28	121	97
Deaths from 60th to 96th days.....	0	4	35	34
Death rate, per cent.....	0	14	29	35
Survivors.....	13	24	86	63



TEXT-FIG. 4. Death rate by 5 day periods among the old and new mice, and combined rate, during the third replacement period.

in Table III. The death rate varied roughly inversely as the length of time the mice had been previously exposed. This higher mortality of 28 per cent is to be contrasted with the lower 14 per cent induced immediately by the replacement. The total mortality arising during the 96 days of the continuance of the experiment involved 114 of 300 mice, or 38 per cent. The death rate for each 5 day period is shown in Text-fig. 4.

Fourth to Tenth Replacements. Experiment 5.—From this point on small additions of new mice were made to the cages and the effects noted. They will not all be recorded in detail although the general effect of the new additions was deaths first among the new and then among the old. Table IV gives the size of each accretion and the mortality attached to each replacement group.

The death rate among the new mice was always in excess of that among the old. In short periods this is to be expected from the general rule that the new mice succumb first. In the table, mice surviving an exposure of 6 to 15 days are regarded thenceforth as old mice, whereas strictly speaking they are neither old nor new. Our impression is that mice exposed to an epidemic over 30 days are to be regarded as old.

TABLE IV.

Deaths among Old and New Mice during the Fourth to Tenth Replacement Periods.

Period.	Duration of period.	Total No. of mice at beginning of period.	Old mice.	New mice.	Deaths during period.			
					Old mice.	Per cent.	New mice.	Per cent.
	<i>days</i>							
4	15	239	198	41	25	13	7	17
5	6	247	207	40	15	7	6	15
6	15	253	226	27	49	18	7	26
7	15	237	197	40	28	14	7	17
8	15	244	214	30	17	8	3	10
9	6	244	224	20	17	8	6	30
10	10	257	227	30	14	6	2	6

The general effect of the addition of the new mice is to maintain the death rate, as Topley has shown. The wave-like character of the fluctuations is evident from Text-fig. 5. The highest rates among the new mice are recorded in the sixth and ninth replacement periods; the interval between the beginning of the frequent replacement series and the sixth is 36 days, and between the sixth and the ninth is likewise 36 days.

Eleventh Replacement, or R 11. Experiment 6.—The effect of the addition of 30 new mice to the 227 old mice of the tenth replacement was negligible, as during 10 days only 1 mouse died; however, within the same time 13 of the old mice succumbed to mouse typhoid. At this point, July 15, 1921, what will be called the eleventh replacement was begun. At the time there were 62 cages in the mouse

village containing 183 mice. Each cage was recruited up to its normal content of 5 animals, thus giving a total population of 310 mice, of which 183 were old and 127 new. The death rate rose sharply among the new mice, and this was succeeded, after an interval of 5 days, by a rise among the old. The higher death rate among the new mice ceased at the 25th day, but later continued at about the same low level as that among the old during the succeeding 50 days (Text-fig. 6). The total mortality from mouse typhoid was 30 per cent, or among the new 31



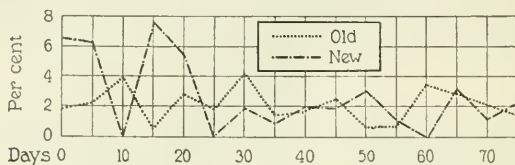
TEXT-FIG. 5. Death rate by 5 day periods among the old and new mice during the fourth to tenth replacement periods, inclusive.

per cent and the old 29 per cent, or as expressed in a proportion of old and new mice succumbing 52:40.

Twelfth Replacement, or R 12. Experiment 7.—October 18, 1921. The twelfth or final replacement was made in such a manner as to give the bacilli ample opportunity to infect new normal mice by retaining a small number of old mice and supplying five times as many new. All of the old mice in Series L and M, and all but 61 of Series K were discarded. To the latter there were added 54 new normal mice, bringing the total of the K series to 115. Series L and M were entirely

replaced by new mice, 100 in each. The three new series, K, L, and M, were now brought together in their usual order. The total number of mice was now 315, of which 254 were new and 61 old remaining from Series K. The ratio of old to new was about 1:4. The series was kept under observation for 60 days with the following results.

2 new mice were killed in fighting on the 1st day, reducing Series K to 113. The total number dying of mouse typhoid infection was 18. Mouse Typhoid II (the epidemic strain) was isolated from 12, and Mouse Typhoid I was isolated from 6. Of the latter, 3 were replacement or new mice.



TEXT-FIG. 6. Death rate by 5 day periods among the old and new mice during the eleventh replacement period.

TABLE V.

Deaths among Old and New Mice during the Eleventh and Twelfth Replacement Periods.

Period.	Duration of period.	Total No. of mice at beginning of period.	Old mice.	New mice.	Deaths during period.			
					Old mice.	Per cent.	New mice.	Per cent.
	<i>days</i>							
11	75	310	183	127	52	29	40	31
12	60	313	61	252	12	20	24	9

The total mortality due to Mouse Typhoid II was therefore 12 out of 113, or 11 per cent, as follows: 9 deaths, or 15 per cent, among the 61 old mice, and 3, or 6 per cent, among the 52 new mice.

Among the 200 mice, all new, in Series L and M, the mortality was 10 and 7 per cent respectively. Mouse Typhoid II was isolated in each instance. The replacement and death figures of Periods 11 and 12 are shown in Table V.

The failure of the addition of new mice to bring about the usual response is doubtless to be explained by the fact that the bacilli were of low virulence.

The deaths due to Mouse Typhoid I call for some explanation. In the preceding series this strain was occasionally isolated, so that it was known to us that it had come in, probably, through stock mice. It is to be supposed that among the 61 old mice taken from Series K there were some carriers of Mouse Typhoid I, and that it was transferred to the new mice added. However, the infection did not spread to the new mice in Series L and M, as Mouse Typhoid II was isolated from each mouse dying in these two series.

The cultures of the spleens from 30 of the mice dying in the twelfth replacement were plated, and from each plate from ten to fifteen colonies were picked. Of the 400 cultures thus obtained, all belonged to Mouse Typhoid II as shown by agglutination with monovalent antiserum.

Mouse Typhoid Carriers.

On September 23, 1921, the total number of surviving mice of Series K, L, and M of the eleventh replacement period was 208, distributed as follows: Series K 79; Series L 70; Series M 69. To them calomel and castor oil were administered, mixed with food so that each mouse received approximately 4 mg. of calomel and 1 cc. of castor oil. The next day portions of the loose stools were easily collected on a platinum loop and cultured. Fourteen positive cultures of bacilli belonging to the *Salmonella* group were obtained. Of the colonies subcultured and tested for agglutination, only one reacted positively with the antiserum of Mouse Typhoid II. The remaining thirteen were not identified immunologically. This result is reported for two reasons, first, as again indicating the occurrence of members of the *Salmonella* group in the intestinal contents of so called normal mice, and second, that the detection of carriers of any particular *Salmonella* organism used for experiment cannot be based on cultural properties alone. Since only five colonies were picked from each plate, it is possible, of course, that the subculturing of many colonies from the stools of the mice found to be positive for *Bacillus typhi murium* might have yielded organisms agreeing immunologically with the strain employed in the experiments.

Immunity.

In a study conducted concurrently with certain of the observations presented in this paper Webster¹⁰ found that mice which had been fed sublethal doses of cultures of *Bacillus typhi murium* were rendered resistant or immune to doses *per os* of the living culture fatal to control mice. This state of increased resistance depended not on local conditions in the intestine but on a general state of immunization attended by the appearance of agglutinins in the blood. It is well known that mice may be protected, at least partially, from parenteral inoculations of the bacillus of mouse typhoid by the injection of killed cultures, and in view of the general immunization thus induced and arising from the ingestion of the bacilli under conditions not leading to death from mouse typhoid, it was deemed desirable to test the survivors of certain of the replacement series with lethal doses of *Bacillus typhi murium* injected intraperitoneally.

Experiment 8.—September 29, 1921. The survivors of the various replacement series L and M, including all periods except R 3, were chosen for the purpose. They numbered in all 128 mice which had been exposed to mouse typhoid from 2 to 18 months before and continued to live in the presumably contaminated surroundings. As controls for this series were taken 10 mice which had been fed 4 months previously on pure-line strains of *B. typhi murium* II without showing any effects or suffering any fatalities, and 40 contact mice in the same series (Q) in which no deaths occurred.²

Each mouse received an intraperitoneal injection of 1 cc. of salt solution containing 1:10,000 of an 18 hour agar slant culture of Mouse Typhoid II. This strain had been injected intraperitoneally into mice and recovered twenty-four times, and in doses of 1:30,000 to 1:10,000 regularly caused death of all mice within 200 hours.

All mice in the test except the 10 fed mice were obviously ill within a short time after injection. The results are shown in Table VI.

The results were decisive and indicate that the surviving exposed mice either have not acquired increased resistance to *Bacillus typhi murium* or do not show it by this method of testing. Judging from the manner of spread of mouse typhoid in the replacement experiments from the new to the old mice, of which some had passed through several successive outbreaks, our inference is that

¹⁰ Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 71.

no definite protection¹¹ is accomplished by long survival in epidemics. However, protection was conferred on the 10 mice which had received large numbers of living bacilli with milk.

TABLE VI.

Results of Intraperitoneal Injection of 1:10,000 of an 18 Hour Agar Slant Culture of Mouse Typhoid into Mice Surviving Epidemic Periods R to R 11.

Mice.	No. injected.	No. dead at 18th day.	Per cent dead.
Survivors of epidemic periods R to R 11	128	108	84
Controls.	40	30	75
Mice previously given large doses of viable single cell strain A by mouth	10	0	0

DISCUSSION.

The results presented in this paper as well as in the first article² of this series can be reproduced regularly under the conditions of the experiments, and thus constitute a succession of events to which the term rule may be applied.

It is perhaps desirable to restate in brief just what the events are which have in common the feature of the exposure of successive batches of mice to a disease, mouse typhoid, initiated purposely in a small number of animals through the ingestion of living mouse typhoid bacilli.

In three separate and distinct experimental attempts to induce outbreaks of mouse typhoid resembling the epidemic spreads sometimes witnessed in breeding stocks and thus spoken of as arising spontaneously, the course of the disease produced was of the nature of a sporadic prevalence. That this preliminary outbreak of disease was of this kind is shown not only by the number of mice dying of it but also by the number of cages attacked.

Once, however, this state of sporadic occurrence of mouse typhoid is inaugurated among a mouse population, all that appears necessary in order to convert the occasional deaths with low cage attack rate

¹¹ Agglutination tests were not made. It is probable that some of the mice possessed blood agglutinins, as it is recalled that in 66 per cent of the tested survivors of the first replacement, the serum agglutinated the epidemic strain.

into frequent deaths with high cage attack rate, is to bring into contiguous relation with the infected population increments of new and healthy mice not previously exposed to the disease.

The particular manner in which the sporadic instances of mouse typhoid arise indicates that very soon a wide distribution of the bacillary incitant takes place in spite of which the fatalities and the cage attack rate remain low.

The sporadic variety of prevalence is soon over. Just what might have taken place subsequently in these lots of mice, had nothing further been attempted, was not determined. No extended observations were made on the survivors, duly segregated, of these preliminary feeding experiments. It can, however, be surmised that under ordinary conditions additional mice would gradually have been added to the population through new births which, in turn, may have become infected, thus reinstating active infection.

But what is definite and significant is the fact that once the sporadic deaths have ceased, the bringing into this population of new lots of mice, which do not mingle directly with the old, suffices to provide the conditions favorable to a wide and even violent outbreak of mouse typhoid, as shown by the fatalities resulting.

The second epidemic spread, or new, in respect to its relative severity and in contrast to the sporadic occurrences, invariably progresses in a certain order. After an interval of about 5 days the new mice begin to succumb, the number of deaths and the proportion of cages attacked rising day by day. During the first period in which the new mice fall victims to the infection, the previously exposed or old mice do not show an increased death rate. But from the 10th to the 20th day following the addition of the new mice and hence the 5th to the 15th day after the new mice begin to succumb, the old mice are drawn into the wave of fatality with results varying somewhat in the different experiments but in which the old mice finally suffer a mortality to an equal, greater, or less degree.

The epidemic spread of the infection, as indicated by the deaths, tends to subside, and even to disappear entirely, before all, sometimes after only a small part, of the exposed mice have been destroyed. A tendency which is obvious in all the experiments is for the establishment of a state of equilibrium between the surviving mice and

the infecting bacillus. This equilibrium is overcome by the introduction of fresh infectible mouse material, resulting in an outbreak of mouse typhoid. The undulations, or epidemic waves, of the disease thus induced in the different replacements are remarkably uniform. The cessation of the undulation, as indicated by the deaths

TABLE VII.

Number of Mice from Preceding Periods Living at the Time of the Twelfth Replacement Period.

Replacement period.	No. of mice living.	Original No.	Deaths.
			<i>per cent</i>
R 1	23	178	87
R 2	3	228	99
R 3	1	114	99
R 4*	17	41	58
R 5	6	40	85
R 6	6	27	78
R 7	8	30	73
R 8	10	30	67
R 9	17	20	15
R 10	21	30	30
R 11	87	127	31

*Replacements 4 to 10 covered short periods of 6 to 15 days each. The total period covered by these seven replacements was 82 days.

is not abrupt but gradual, although the late fatalities may not be due to delayed infection so much as to protracted illness and long survival.

The experiments show definitely that mice which have weathered the storm of the epidemic in the sense of having passed, without succumbing, through one or more violent outbreaks of the disease, are not insured against eventual fatal attack. This fact is brought out in Table VII, which shows that as successive epidemic waves pass over the always changing population, the old or previously exposed mice tend ultimately to be wholly wiped out.

In due time the precise conditions concerned in the conversion of the incipient and sporadic into epidemic spreads of disease will need to be considered narrowly. We have learned in respect to the enteric infection mouse typhoid how the one may be turned into the other

and that the epidemic outbreaks are always, as it were, frustrated before all the available infectible material is consumed.

Obviously there are two outstanding factors which affect and determine the state of the microbic incitant in its relation to the host: the one relates to the quality or virulence, so called, the other to the quantity or number. Hitherto it has been the virulence factor that has been most considered and invoked to account for the wave-like movement of epidemic disease. This fact will appear prominently in the brief review of the literature which is to follow. The second or quantity factor has been much less discussed. As pointed out in the body of this paper, we believe that it cannot well be disregarded. Perhaps the disease mouse typhoid may provide favorable experimental materials for just this study. Our experiments show decisively that immediacy of contact is by no means a necessary condition for the wide diffusion of the microbic incitant of mouse typhoid among a mouse population maintained in small segregated groups; and this fact is emphasized also by the occurrences of the spontaneous epidemic described by Lynch.¹ The mere rapidity of the fluctuation of the curve of the death rate in the replacement experiments either indicates that the virulence factor is highly unstable, or that some other attending condition acts to modify or control the results as measured by infection and death.

Thus far attention has centered on the factors affecting the microbic incitant, while those which may be presented by variation in the host have yet to be considered. Difficult as may be the unravelling of the former, for which certain methods possibly are at hand, the other has been regarded next to insuperable. Aside from choosing a stock roughly homogeneous and covering the matter of size and age, nothing has been attempted as regards the host. In a recent study¹² of tuberculous infection in guinea pigs an effort has been made to obtain values for certain hereditary qualities, an aspect of the problem of infection in mouse typhoid capable of treatment.

The relation of the carrier state to the host awaits elucidation. That carriers arise among the exposed mice which do not succumb early is demonstrated. Experience indicates that the detection

¹² Wright, S., and Lewis P. A., *Am. Naturalist*, 1921, lv, 20.

of carriers is determinable not by cultivation tests alone but chiefly through the reactions of immunity. Without the latter, error readily creeps into the results because of the presence in the intestine of apparently normal mice, of members of the broad group of organisms embraced under the loose term of mouse typhoid bacilli.¹⁰ Webster's experiments show that the purposive introduction of living or even killed mouse typhoid bacilli into the intestines of normal mice, under proper conditions, leads to an immunization sufficing to prevent infection from otherwise fatal infecting doses of cultures for such mice. This state of immunity is general in the sense that specific agglutinating bodies are demonstrable in the blood, and the immunized mice resist not only intrastomachic but also intraperitoneal injections of the living bacilli.

Review of the Literature.

The possibility of employing artificial epidemics among small rodents for the study of the spread of infection should date from the experiments of Danysz,¹³ published in 1900. As will be recalled, he endeavored to exterminate rodents, rats especially, on a large scale through the employment of living cultures of a bacillus belonging to the so called mouse typhoid group. The method did not really succeed. The reasons for the failure are now apparent and, in the main, arise first from the rapid loss of infecting power or virulence of the cultures, and second from the circumstance that the bacillus as it occurs in the rat cadaver is less infective than at the moment infection takes place. Hence the devouring of the cadavers by their living companions arrests rather than propagates the disease. These unsuccessful results were foreshadowed by laboratory studies made by Danysz, who found that no matter how highly active the cultures were at the outset, their power of infection by feeding did not extend beyond a very few passages. Danysz' conclusions may be stated as follows:

Cultures may be enhanced so that they will induce fatal infection on ingestion, but the increased activity will not endure beyond the third or fourth passage; exposure of a group of non-infected mice to a small number of mice purposely fed on a virulent culture will induce fatal infection in most but not in all of the former. The few survivors succumb to a later direct feeding of culture of average virulence. Cultures enhanced by organ and blood passages suffer concomitant diminished activity when administered *per os*. Cultures obtained from the blood and organs in the incubation stage of infection are more virulent than when derived

¹³ Danysz, J., *Ann. Inst. Pasteur*, 1900, xiv, 193.

from cadavers. Resistance or susceptibility to infection among small rodents is affected by race and age, and even by individual peculiarities.

Later, Bainbridge¹⁴ noted that a spontaneous epidemic among wild rats kept under experimental conditions may lead to the death of all, and as the conditions approach those of nature, the death rate becomes less. The percentage of deaths noted by Bainbridge was not affected by the number of rats devoured, and he considered the rat to possess a preexisting immunity which arises through the occurrence of spontaneous epidemics among those animals. In support of this view, rats surviving the feeding of cultures were found to carry agglutinating properties in their blood for the bacilli used.

The conditions favoring the perpetuation of the pathogenic proclivities of the mouse typhoid bacilli have been investigated recently by Topley. He has utilized his material, as we had planned to use ours, in an endeavor to penetrate into the hidden recesses of epidemiology. He repeated Danysz' experiments, which he confirmed, but he went beyond the latter in acting on the supposition that the consumption of food soiled by excreta may be a more common and effective mode of infection than the devouring of cadavers carrying already depressed bacilli.

From the first, Topley¹⁵ looked upon the spread of epidemics and their rise and fall as having to do primarily and essentially with the infecting power of the inciting microbe. The rapid fluctuations of the mouse typhoid bacillus in this regard seemed to provide the needed explanation of the failure to presage epidemics among mice. He sought to supply the needed conditions by starting an epidemic through feeding and then adding, in a large cage, daily small complements of normal mice, in order, through passage, to keep the bacilli alive and actively infective. In this way the tendency for the disease, once started, to cease abruptly was overcome, and the infection would proceed in regularly recurring waves, apparently without time limit. He further observed that the deaths occur in large groups with intervals between, during which the deaths are few or far between. He distinguished two phases according to which the new mice added tended to succumb or survive, and these phases are the rise of a wave when infection is likely to occur and its fall when it is likely to fail. Finally, he noted that mice which have passed successfully through one epidemic wave may succumb to a later one, and an infected population to which no additions are made will outlive one into which fresh mice are brought, while the ultimate survivors have not escaped infection and a considerable part has become carriers. A substitution of strains took place during Topley's experiments as in ours. He began with a strain of Gärtner's bacillus, but as his experiments progressed a second organism, identified as belonging to the *suipestifer* group, intervened. Subsequently strains

¹⁴ Bainbridge, F. A., *J. Path. and Bact.*, 1909, xiii, 443.

¹⁵ Topley, W. W. C., *J. Hyg.*, 1920-21, xix, 350.

of Gärtner's bacillus, or of this second organism, or both together, were obtained from the great majority of the dead animals examined.¹⁶

In a second communication¹⁷ the outbreak of an epidemic among a normal stock, probably through accidental spread from cages with infected mice, is described. Not all the exposed mice succumbed and the deduction made was that the infection was not of "uniform intensity, passed from a healthy carrier to one or more susceptible individuals, but that some process is set in motion, which results in an increase in the infectivity of the parasite, this in its turn giving rise to a fresh wave of mortality among the cage population."

Topley's experiments were arranged to achieve the greatest likelihood of infection occurring by providing the greatest possible concentration of contaminated materials; namely, dejecta carrying the bacilli or cadavers devoured by their companions. Our experiments were long under way before Topley's main publications appeared in 1921. We had in mind not the providing of optimal conditions for infection to take place in mice, but the imitation, if only roughly, of those occurring naturally in man and in laboratory animals, in connection with which epidemics of disease occur.

SUMMARY.

1. A kind of mouse village was set up into which was introduced a small number of mice fed on a culture of so called mouse typhoid (*Bacillus pestis caviæ* of the *Bacillus paratyphosus* B group) bacillus. The spread of the infection so induced to the cages, or "homes," of the other mice was left to accident through the attendant who fed the animals and cleaned the cages. That this means was likely to be sufficient was deduced from the epidemic reported by Lynch.¹ A spot map was kept throughout the experiments which extended from 1919 into 1922.

2. The first effect of the exposure of normal mice to a much smaller number of mice fed on the culture is to set up a *sporadic*,

¹⁶ Topley and his coworkers (Topley, W. W. C., Weir, H. B., and Wilson, G. S., *J. Hyg.*, 1921, xx, 227) have studied these two organisms serologically, along with other related bacilli, and suggest that the relations between the Gärtner's bacillus and *B. suispestifer* group of organisms is so close that for purposes of classification and nomenclature the name of *B. enteritidis* may, for convenience, be applied to the whole group.

¹⁷ Topley, W. W. C., *J. Hyg.*, 1921, xx, 103.

not an *epidemic* outbreak of mouse typhoid. This is the regularly recurring incident of the experiment as shown by low mortality and low cage attack rate. Such a sporadic prevalence is self-limited in time.

3. The introduction of fresh, normal mice into a community in which sporadic deaths are occurring leads regularly, not to the further extension of the sporadic deaths, but to an epidemic spread, as shown by high mortality and high cage attack rate. The epidemic begins with deaths among the new mice, but extends to the old mice which succumb later. The spread ceases and the wave subsides before all the mice have succumbed. A state of equilibrium between the infecting bacillus and the surviving mice is reached; no more deaths occur. The epidemic outbreak, therefore, is also self-limited in time.

4. If, now, another new addition of normal mice is brought into the potentially infected community, the events are reenacted; deaths occur among the new, another epidemic wave sweeps through the population, again claiming victims among the previous survivors.¹⁸ Through the replacement of the destroyed mice with fresh, normal mice, epidemic wave after wave is produced, until certain groups of old survivors are entirely wiped out. There seems to be no limit to this process, as there will always be survivors at least of the later groups added.

5. The dying down of the epidemics and the attaining of the equilibrium do not mean the elimination of all the bacilli. Potential infection still lurks in mouse "carriers" and on the hands of the attendant. No successful method of completely removing the bacilli from the hands of the attendant was found.

6. The maintaining of the epidemic waves is dependent on the presence of new lots of mice, whether supplied from without or produced within through new births. It is the latter sources which provide the consumable material in such natural epidemics as that described by Lynch.¹ Hence their slower movements as compared with the epidemic spreads and rise and fall of the epidemic waves in the artificially propagated instances in which new batches of mice are brought into the village in bimonthly intervals.

¹⁸ In the third replacement period a rise in death rate constituting a small epidemic occurred after the 60th day and independent of the addition of new mice. No explanation has been found for this occurrence.

7. The evidence at hand is to the effect that the degree of infectivity of "mouse typhoid" bacilli is highly fluctuating, and it appears that all the bacilli which are included under that name, classed variously as *Bacillus enteritidis*, Gärtner's bacillus, *Bacillus paratyphosus* B, *Bacillus suispestifer*, and *Bacillus pestis caviae*, infect mice in a similar, possibly indistinguishable manner, inducing self-limited outbreaks of disease reaching at times epidemic proportions. This quality of infectivity, or virulence, is one factor in the process but alone does not suffice to account for the observed facts. A second influence is not improbably quantity, or dosage, of the inciting micro-organism. Attention to the manner of spread of an epidemic, as revealed graphically by the spot map, shows that it is never uniform but patchy. The supposition is, therefore, that among the new mice are certain individuals so highly susceptible as to react to small numbers of bacilli of average infectivity. Within these animals the multiplication is rapid, so that a wider spread of much larger amounts of these average, or even temporarily enhanced bacilli takes place with the inevitable effect of communicating, through greater dosage, etc., the infection to other and less susceptible individuals among the new and also the older lots. This process continues so long as rapid multiplication can occur. This latter is, in turn, determined by the innate tendency of the bacilli to return to an average of infectivity and by mouse individuals of greater resistance to restrict free growth and multiplication. In this manner, possibly, the epidemic spread is checked and the curve representing it made to fall more or less quickly to a base-line.

EXPERIMENTS ON NORMAL AND IMMUNE MICE WITH A BACILLUS OF MOUSE TYPHOID.

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As part of a wider study of the phenomena of epidemics among animals described by Flexner¹ and Amoss,² the following series of experiments was undertaken to ascertain the varieties and degrees of resistance in normal and immunized mice to mouse typhoid bacilli of the paratyphoid-enteritidis group. The experiments were so designed as to bear directly on such questions as the relation of infectivity on the part of the microorganisms to the portal of entry into the body of the host and also on the influence of a possible local as opposed to a general immune state, the effect of which would be to alter or even to abate the danger of infection by the ordinary route traversed in nature.

Since the problem of infection, as manifested in the higher animal species, is a complex made up of potentialities in the host and in the microbe, it becomes necessary to control and keep constant as many of the factors as is possible. Hence the mice, chosen from healthy stock with practically no death rate, were of one age, often from the same litter, of practically the same weight, and were kept under close observation before and during experiments. The stock room diet of bread and milk was continued throughout the experiments. The mouse typhoid strain, M. T. II, obtained from an experimental epizootic studied by Amoss,² was identified with *Bacillus pestis caviæ* Smith and closely related to *Bacillus aertrycke* (mutton) of Schütze.³ This organism was carried in stock and maintained by monthly stab cultures in tube agar of pH 7.4. In order to secure a culture of

¹ Flexner, S., *J. Exp. Med.*, 1922, xxxvi, 9.

² Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 25, 45.

³ Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 97.

Strain M. T. II of a degree of virulence adequate to infect regularly *per os*, it was necessary to pass it through a series of mice by intrastomachal inoculation. At autopsy, to guard against substitution, the identity of the recovered bacilli was always established by means of fermentation and agglutination tests. Dosage was determined by making use of the fact that cultures of Strain M. T. II in plain broth of pH 7.4, when inoculated in small amounts, reached, after 14 to 24 hours incubation at 37°C., a relatively constant number; e.g., 1 billion per cc. With a 14 to 24 hour broth culture the approximate number of organisms contained in any dilution could thus be calculated.

Effects on Normal Mice of Intrapleural, Intraperitoneal, and Intrastomachal Inoculations.

Intrapleural Inoculation with Culture M. T. II.—These experiments and others to follow were planned in order to ascertain the manner of resistance of mice to intrapleural injections of the cultures. The tests were made as follows:

9 mice with controls were given intrapleurally 0.2 cc. of a 16 hour broth culture of Strain M. T. II obtained from the stock agar tube. Interpreting these and all subsequent dilutions on the 1 cc. basis, 3 mice received 1:5,000 dilution, 3 received 1:50,000 dilution, and 3 received 1:500,000 dilution. At 2 hour intervals small amounts of fluid were aspirated from the infected pleural cavity. Films were made and 1 drop was transferred into fluid agar (48°C.), shaken, and poured. Colonies were counted after 24 hours incubation (37°C.). Table I summarizes the duration of life of experimental animals; the controls ran a similar course.

TABLE I.

Duration of Life of Mice Injected Intrapleurally with Strain M. T. II

Dilution.	Duration of life in Experiment 2.	Duration of life in Experiment 3.	Duration of life in Experiment 4.	Average duration of life.
	<i>days</i>	<i>days</i>	<i>days</i>	<i>days</i>
1:5,000	5-1-3	1-6-3	1-1-3	2+
1:50,000	5-5-6	Survived*-3-5	4-10-7	5+
1:500,000	6-7-5	15-9-14	10-6-6	8+

* Survivors were discarded after 30 days.

The counts of the colonies on the plates, although not quantitatively accurate, were, nevertheless, of value in showing the relative changes in numbers. Such sources of error as dry puncture, irregularity of distribution of bacilli in the pleural exudate, and variation in size of drop, did not obscure the tendency of the bacteria after an initial lag of 4 to 6 hours to increase rapidly in number until the mice were overwhelmed (Table II and Text-fig. 1).

TABLE II.

Bacterial Growth in Vivo (Intrapleural).

Dilution 1:50,000; number of colonies in plate 150 to 200.

Length of time after injection. hrs.	Mouse 1.				Mouse 2.				Mouse 3.			
	Condi- tion.	Smear.		No. of colo- nies in plates.	Condi- tion.	Smear.		No. of colo- nies in plates.	Condi- tion.	Smear.		No. of colo- nies in plates.
		Cells.	Phago- cytosis.			Cells.	Phago- cytosis.			Cells.	Phago- cytosis.	
1	Well.	±	0	100	Well.	±	0	30	Well.	±	0	60
2	"	±	0	100	"	±	0	50	"	±	0	100
3	"	±	±	50	"	0	0	50	"	0	0	40
4	"	±	+	100	"	0	0	100	"	0	0	100
5	Sick.	+	+	150	Sick.	+	+	250	Sick.	0	0	15*
6	"	+	?	250	"	+	+	250	"	?	?	250
24	"	++	+	750	"	+	+	250	"	+	?	150
48	"	++	+	750	"	+	?	250	"	?	?	250
72	"	++	+	1,200	"	++	+	720	"	+	+	450
98	"	?	?	1,620	"	+	?	690	"	+	+	2,000
119	Dead.				Dead.				Dead.			
143												

* Probably an error.

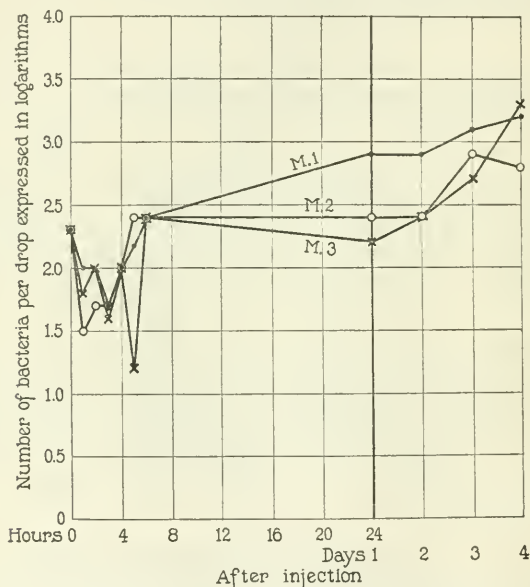
The series of events following the intrapleural injection of fixed doses of a constant culture was quite regular, as was shown by repetition of the experiment just described.

Intraperitoneal Inoculation with Culture M. T. II.—This experiment was the counterpart of the pleural one immediately preceding. It was carried out as follows:

6 mice with controls were injected intraperitoneally with 0.2 cc. of a 16 hour broth culture of Strain M. T. II obtained from the stock agar tube, 3 receiving 1: 5,000 and 3, 1:50,000 dilution. At short intervals small amounts of fluid were

aspirated from the peritoneal cavity. Films were made and 1 drop of the exudate was plated in the fluid agar (48°C.) medium. The colonies were counted after 24 hours incubation (37°C.).

The result is similar (Table III); after an initial lag, the bacilli multiply rapidly until they reach a high number at about the time death occurs, as is shown in Text-fig. 2.



TEXT-FIG. 1. Bacterial growth *in vivo* (intraleural). The figures are taken from Table II.

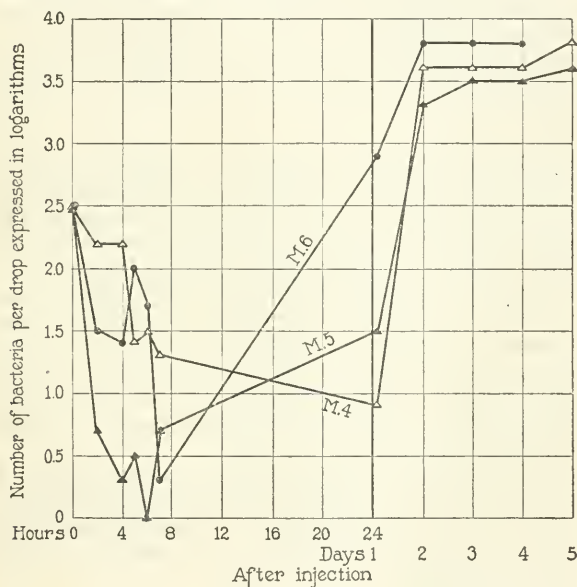
The events in the two experiments with pleural and peritoneal inoculation can be summarized as follows: if live cultures of this organism are injected intrapleurally or intraperitoneally into normal mice, there occurs an initial lag in the rate of bacterial multiplication lasting 4 to 6 hours, followed by a rapid and continual acceleration of

TABLE III.

Duration of Life of Mice Injected Intraperitoneally with Strain M. T. II.

Dilution.	Duration of life in Experiment 9.	Duration of life in Experiment 10.	Average duration of life.
	days	days	days
1:5,000	6-3-5	10-10-Survived.*	7
1:50,000	7-6-5	13-5-13	8+

* Survivors were discarded after 20 days.

TEXT-FIG. 2. Bacterial growth *in vivo* (intraperitoneal).

growth until the death of the animal. To this rule there are exceptions in an occasional recovery or an undue prolongation of the survival period. The mice dying acutely exhibit few pathological changes,

while those in which the infection pursues a more chronic course show macroscopically thick peritoneal or pleural exudation, and lesions of the lungs, liver, and spleen consisting of hepatization, nodule formation, swelling, etc. The injected bacillus was always recovered from the heart's blood and organs.

Intrastomachal Inoculation with Culture M. T. II.—A fixed dose which ranged from 0.5 to 1 cc. of the broth culture diluted 1:100 was injected into the stomach through a stiff silver catheter attached to a suitable measuring syringe with rubber tubing. This slight procedure which assures ingestion of the culture can be carried through expeditiously and securely. Culture M. T. II could be kept at an approximately constant infecting level if passed through mice *per os* at frequent intervals. The injections *per os* were made about 3 hours after feeding.

Thus far, in spite of the control exercised over the origin and selection of the mice and the uniform treatment and dosage of the culture, some degree of fluctuation has attended the experiments in infection of normal mice *per os*. That irregularities would enter into this part of our studies was of course foreseen. It is highly probable that under ordinary conditions of propagation of an epizootic among mice by a representative of the paratyphoid-enteritidis group, infection takes place always *per os*, and yet we know that survivors invariably occur. Our experiments show that the survivors have no necessary relation to dosage of a given constant culture, although on the whole the animals receiving the larger doses are the ones tending to succumb. Table IV is intended to bring out the variations met with in a number of carefully planned tests.

In addition to the irregularities shown in these experiments, it happens that still greater ones are met with when instead of introducing the culture directly into the stomach of mice, the organisms are added to the food by soaking up the broth culture with bread which is then fed to the animals.

For this test the mice were assembled 6 per cage, and it was noted that they devoured the soaked bread voraciously on successive days without necessarily succumbing to infection. Table V summarizes the results of two such feeding experiments.

TABLE IV.

Variation in Susceptibility Following Injection per Os (Stomach Tube).

No. of mice.	Broth dilution.	Duration of life in fatal cases.	No. of survivors.	Result.
		<i>days</i>		
1	1:100	9	0	Regular.
2	1:200	12-16	0	
1	1:500		1 (60 days).	
3	1:100	8-14-20	0	Regular.
1	1:1,000		1 (60 days).	
1	1:10,000		1 (60 ").	
3	1:100	19-21	1 (60 ").	Regular.
1	1:1,000		1 (60 ").	
1	1:10,000		1 (60 ").	
3	1:50	12-43	1 (60 ").	Irregular.
3	1:500	11	2 (60 ").	
3	1:5,000	17-18-23	0	
3	1:50,000		3 (60 days).	
2	1:1	18	1 (30 ").	Irregular.
3	1:10	8-11-21	0	
3	1:100	7-8	1 (30 days).	
2	1:1	4-10	0	Irregular.
2	1:10		2 (18 days).	
2	1:100	17-18	0	

TABLE V.

Variation in Susceptibility Following Injection per Os of Strain M. T. II (with Feedings).

No. of mice.	Duration of feeding.	Broth dilution.	Duration of life in fatal cases.	No. of survivors.	Result.
	<i>days</i>		<i>days</i>		
3	3	1:10,000	12	2 (30 days).	Irregular.
3	5	1:10,000		3 (30 ").	
3	7	1:10,000	17	2 (30 ").	
5	5	1:10,000		5 (40 ").	Irregular.
5	7	1:10,000	11-11-17	2 (40 ").	
5	9	1:10,000	15	4 (40 ").	
5	13	1:10,000	22-38	3 (40 ").	

Although the stomachal injections and the feedings of broth cultures of Strain M. T. II did not consistently induce infection but rather brought out factors of variation, themselves illuminating when considered in relation to observed epizootics arising accidentally among mice, yet certain general features may be gleaned from the tests performed. An incubation period of about 5 days preceding the indications of infection or death was noted. There was no invariable relation between dosage and fatal infection; without reference to dosage or culture certain mice proved refractory. The type of infection was predominatingly chronic with regular involvement of the intestine, spleen, and liver, and occasional involvement of the lungs and heart. In all instances Strain M. T. II was recovered from the heart's blood and organs.

Effects on Immune Mice of Intrapleural, Intraperitoneal, Subcutaneous, and Intrastomachal Inoculations.

The next tests related to the influence exerted by immunizing injections of dead or living bacilli on the subsequent inoculation of given doses of active cultures of Strain M. T. II. The experiments were arranged to bring out the effect not only of the immunizing action in general immunity but also of a possibly greater state of local immunity at the site of the protective inoculations. The live organisms were introduced into the pleural and peritoneal cavities and also into the stomach.

The killed cultures employed for the protective injections consisted of a 24 hour broth culture which had been heated to 55°C. for 2 hours and afterwards standardized and preserved with 0.3 per cent tricresol.

Intrapleural and Intraperitoneal Inoculation.—The killed bacilli, in doses of 250,000, were injected either into the right pleural or the peritoneal cavity, after which, at the expiration of a given period of time, living bacilli were injected into one or the other of these cavities, as shown in the protocols which follow.

12 mice were inoculated with 250,000 killed Strain M. T. II bacilli, 6 intrapleurally and 6 intraperitoneally, and 7 days later the inoculations were repeated. 9 days after the second inoculation the mice were divided into batches of 3 and injected with 0.2 cc. of a 1:1,000, 14 hour broth culture of Strain M. T. II either intrapleurally or intraperitoneally. 3 pleural "vaccinated" mice, along with 3

normal controls, were injected intrapleurally, another 3 of the pleural "vaccinated" mice, together with 3 controls, received the material intraperitoneally. At intervals, fluid was aspirated from the cavity into which the living bacilli were injected, films were made, and 1 drop was plated in agar. The colonies developing in the latter were counted after 24 hours growth. Tables VI to VIII inclusive and Text-fig. 3 give in brief the results of the experiment.

TABLE VI.

Duration of Life of Normal and "Vaccinated" Mice Injected Intrapleurally and Intraperitoneally.

Mice.	Mice "vaccinated" intrapleurally; injected intrapleurally.		Mice "vaccinated" intrapleurally; injected intraperitoneally.	
	Duration of life.	Average duration of life.	Duration of life.	Average duration of life.
	days	days	days	days
"Vaccinated".....	13-14-28	18+	14-17-25	18+
Controls.....	2-4-4	3+	1-1-5	2+

TABLE VII.

*Bacterial Growth in the Pleural Cavities of Normal and "Vaccinated" Mice.
Mice "Vaccinated" Intrapleurally; Injected Intrapleurally.*

14 hr. broth culture of Strain M. T. II; dilution 1:5,000; 5,000 organisms per drop.						
Length of time after injection.	Mouse 1, "vaccinated." No. of colonies in plates.	Mouse 2, "vaccinated." No. of colonies in plates.	Mouse 3, "vaccinated." No. of colonies in plates.	Mouse 4, control. No. of colonies in plates.	Mouse 5, control. No. of colonies in plates.	Mouse 6, control. No. of colonies in plates.
<i>hrs.</i>						
2	2	0	1	100	420	120
3	0	0	0	300	150	300
5	0	0	5	450	600	60
6	3	0	3	450	180	800
8	0	0	1	450	3,600	720
9	0	0	0	4,000	5,400	3,000
30	120	0	30	2,000	900	800
<i>days</i>						
2	0	0	10	{ 5,000 Dead.	100	1,800
3	0	3	2		360	Dry tap.
4	180	40	10		Dead.	Dead.
5	240	25	150			
11	1,620	1,080	1,350			
13	Dead.					
14		Dead.				
23			1,440			
28			Dead.			

TABLE VIII.

*Bacterial Growth in the Peritoneal Cavities of Normal and "Vaccinated" Mice.
Mice "Vaccinated" Intrapleurally; Injected Intraperitoneally.*

Length of time after injection.	14 hr. broth culture of Strain M. T. II; dilution 1:5,000; 5,000 organisms per drop.					
	Mouse 7, " "vaccinated." No. of colonies in plates.	Mouse 8, " "vaccinated." No. of colonies in plates.	Mouse 9, " "vaccinated." No. of colonies in plates.	Mouse 10, control, No. of colonies in plates.	Mouse 11, control, No. of colonies in plates.	Mouse 12, control, No. of colonies in plates.
<i>hrs.</i>						
2	90	1	10	360	3,600	80
3	0	0	15	3	1,350	550
5	4	0	10	40	720	15
6	1	0	1	120	1,080	1,350
8	0	0	0	3,150	4,000	6,000
9	0	2	0	4,000	6,000	6,000
30	15	0	30	4,000	Dead.	Dead.
<i>days</i>						
2	20	0	Dry tap.	4,000		
3	0	15	" "	6,000		
4	150	360	" "	6,000		
5	180	90	" "	Dead.		
11	6	3,000	" "			
14		Dead.				
17	Dead. ^a					
23			15			
25			Dead.			

TABLE IX.

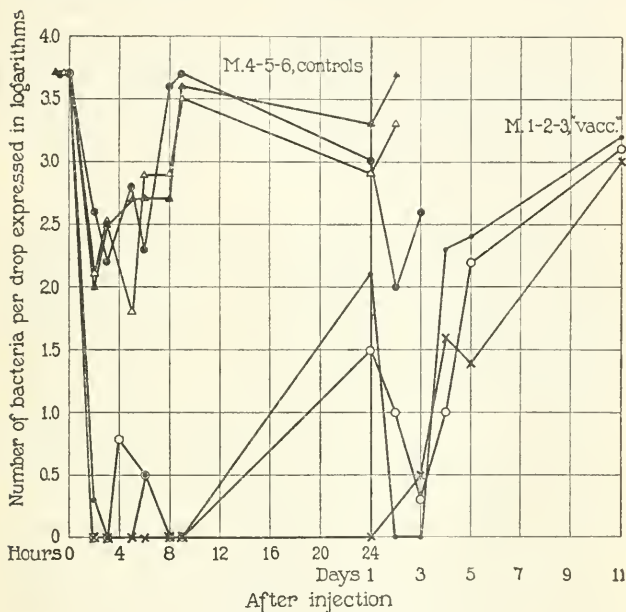
Duration of Life of Normal and "Vaccinated" Mice Injected Intrapleurally and Intraperitoneally.

Mice.	Mice "vaccinated" intraperitoneally; injected intrapleurally.		Mice "vaccinated" intraperitoneally; injected intraperitoneally.	
	Duration of life.	Average duration of life.	Duration of life.	Average duration of life.
	<i>days</i>	<i>days</i>	<i>days</i>	<i>days</i>
"Vaccinated."	15-17-19	17	21-36-Survived.*	32+
Control.	4-4-1	3	3-4-4	3+

* Survivors were discarded after 40 days.

This experiment was paralleled by a similar one in which the "vaccination" had been made intraperitoneally, followed as before by the injection of living bacilli into the pleural or peritoneal cavity. The results in general were identical

with the preceding: Table IX shows the duration of life in the series and the curves illustrating bacterial growth in "vaccinated" mice and controls are similar to those of Text-fig. 3.



TEXT-FIG. 3. Bacterial growth in the pleural cavities of normal and "vaccinated" mice.

The experiments may be summarized as follows: Living bacilli injected into the control mice showed the usual lag of 4 to 6 hours, followed by a rapid increase in number until the death of the animal. These mice succumbed within 3 days after injection, showed few lesions at autopsy, and positive cultures from the heart's blood and organs. Living bacilli injected into the "vaccinated" mice were partially destroyed and held in check by the protective mechanism of

the animal body for 2 or 3 days. Subsequently the rate of bacterial multiplication increased gradually until the death of the animal. These mice succumbed within 18 days after injection, and showed at autopsy extensive local lesions as well as marked involvement of the spleen and liver. Cultures from the heart's blood, exudates, and organs were positive. The partial immunity following this type of treatment is entirely of a general nature; no essential difference in the progress of infection or ultimate protection is to be noted whether the mice are given the injection into the cavity which previously received the "vaccine" or into the cavity previously "unvaccinated."

Subcutaneous Inoculation.—

As a corollary to the above, 6 mice were "vaccinated" subcutaneously with a total of 150,000,000 bacilli given in two doses. 5 days after the second inoculation, twice the usual dose of living bacilli was given to 3 of the mice intrapleurally, 3 intraperitoneally, and to normal controls. Because of the larger dose of living bacilli, the controls died within 24 hours, while the average survival period of the "vaccinated" mice was 17 days. The progress of the infection, however, was to all intents and purposes identical with that in the mice "vaccinated" by way of the pleura and peritoneum and their controls.

Inoculation per Os.—The ordinary, or as we say, natural mode of infection with mouse typhoid bacilli is by way of the gastrointestinal tract and hence the influence of the "vaccination" on infection *per os* was next studied. Since the preceding tests all indicate that the effects of protective inoculation with killed cultures on the progress of a subsequent injection of living bacilli are practically identical, however the "vaccination" is carried out, namely by the pleural, peritoneal, or subcutaneous route, the inoculation of dead bacilli in this experiment was made subcutaneously.

150 normal mice of approximately the same weight (16 to 18 gm.) and age (2 months) were segregated. To 120 of them 0.5 cc. of a 24 hour broth suspension of *Bacillus M. T. II*, previously heated to 55°C. for 2 hours, was given under the skin. The "vaccine," preserved with 0.3 per cent tricresol, was standardized to contain 500,000,000 bacilli per cc., so that each mouse received an initial dose of approximately 250,000,000 dead bacilli. 30 mice were set aside to be used as controls.

After an interval of 13 days, 10 of the mice having received one injection of the "vaccine" were given an intraperitoneal injection of 0.5 cc. of a living 14 hour broth culture of Mouse Typhoid II, diluted 1:100, and a second 10 of the "vaccin-

ated" mice received the same dose into the stomach by silver tube. Controls were injected intraperitoneally and *per os* with doses of 0.5 cc. of the same broth culture diluted 1:50 to 1:250.

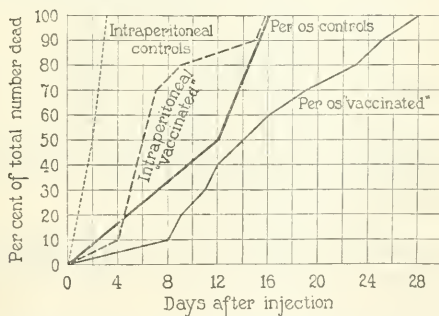
TABLE X.

Duration of Life of Normal and "Vaccinated" Mice Injected Intraperitoneally and Intrastomachally.

Mice.	No. of mice.	Dosage.	Duration of life.	Average duration of life.
			days	days
Intraperitoneal control.	1	1:100	1	1
" " "	2	1:200	2-3	2.5
" " "	1	1:500	4	4
" " "vaccinated."*	10	1:200	4-5-5-6-6-7-7-9-15-16	8
<i>Per os</i> control.	1	1:100	9	9
" " "	2	1:200	12-16	14
" " "	1	1:500	Survived.†	
" " "vaccinated."*	10	1:200	8-9-11-12-14-16-19-23-25-28	16.5

* The "vaccinated" mice had received one subcutaneous injection previous to the intraperitoneal or intrastomachal injection.

† Survivors were discarded after 62 days.



TEXT-FIG. 4. Duration of life of "vaccinated" mice injected intraperitoneally and intrastomachally. The figures are taken from Table X.

Table X and Text-fig. 4 summarize the results of this experiment, which can be expressed as follows: A single subcutaneous inoculation

of killed bacilli into mice confers a partial protection against subsequent introduction of live organisms *per os* as well as by way of the peritoneal cavity.

The remaining 100 "vaccinated" mice received a second subcutaneous injection of the killed cultures 14 days after the first. After the expiration of another 18 days, 20 of these mice were given 1 cc. of a 14 hour living broth culture of Strain M. T. II diluted 1:100 into the peritoneum, and 20 the same dose by stomach tube. Table XI and Text-fig. 5 summarize the results of the injections in these animals and in the controls.

TABLE XI.

Duration of Life of Normal and "Vaccinated" Mice Injected Intraperitoneally and Intrastomachally.

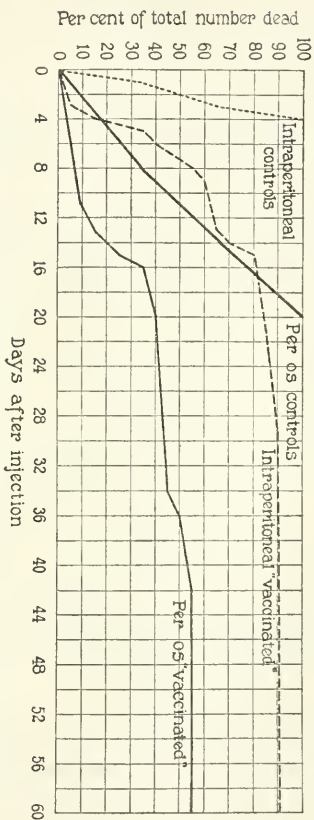
Mice.	No. of mice.	Dosage.	Duration of life.	Average duration of life.
			<i>days</i>	<i>days</i>
Intraperitoneal control.	3	1:100	1-3-4	2.6
" "	1	1:1,000	5	5
" "	1	1:10,000	5	5
" "vaccinated."*	20	1:100	3-4-4-5-5-5-5-6-8-8-8-9-13-14-15-15-21-29. Two survivors.†	10
<i>Per os</i> control.	3	1:100	8-14-20	14
" "	1	1:1,000	Survived.†	
" "	1	1:10,000	" †	
" "vaccinated."*	20	1:100	11-11-13-15-15-16-16-20-34-36-42. Nine survivors.†	20

* The "vaccinated" mice had received two subcutaneous injections previous to the intraperitoneal or intrastomachal injection.

† All survivors were discarded after 62 days.

The increase in partial protection following a second injection of "vaccine" is effective against the *per os* as well as the peritoneal portal of entry.

40 of the remaining "vaccinated" mice were given 25 days later a third subcutaneous injection of 250,000,000 killed bacilli, of which, after the lapse of another 10 days, 20 were injected into the peritoneum and 20 by stomach tube with the regular dose of 1 cc. of a 14 hour living broth culture diluted 1:100.



TEXT-FIG. 5. Duration of life of "vaccinated" mice injected intraperitoneally and intrastomachally. The figures are taken from Table XI.

The results are given in Table XII and differ from those in Table XI only in the number of survivors among the "vaccinated" animals, which is much larger. It must be noted, though, that the increased protection from the third "vaccination" is somewhat more effective against the introduction of live organisms intraperitoneally than by way of the mouth.

The results of these experiments show that mice "vaccinated" subcutaneously with a certain mouse typhoid strain are partially

TABLE XII.

Duration of Life of Normal and "Vaccinated" Mice Injected Intraperitoneally and Intrastomachally.

Mice.	No. of mice.	Dosage.	Duration of life.	Average duration of life.
			<i>days</i>	<i>days</i>
Intraperitoneal control.	3	1:100	1-1-1	1
" "	1	1:1,000	1	1
" "	1	1:10,000	5	5
" "vaccinated."*	20	1:100	7-8-8-10-10-12-12-13-15-16-17-18-32-33. Six survivors.†	15+
<i>Per os</i> control.	3	1:100	19-21. One survivor.†	20
" " "	1	1:1,000	Survived.†	
" " "	1	1:10,000	" †	
" " "vaccinated."*	20	1:100	8-10-10-12-18-18-21-34. Twelve survivors.†	17

* The "vaccinated" mice had received three subcutaneous injections previous to the intraperitoneal or intrastomachal injection.

† All survivors were discarded after 62 days.

protected against a subsequent introduction of the homologous live organisms intraperitoneally or *per os*, and that each dose of "vaccine" seems to confer a similar relative increase in resistance to the peritoneal or gastrointestinal route of infection. Again, given a fixed dose of this culture, it is clear that intraperitoneal injection into normal mice produces an acute type of disease, while the intrastomachal route is followed by a more chronic infection. It would appear, then, that a short interval of time is consumed by the organism in overcoming the

natural defenses of the peritoneal cavity and that a longer period of time is necessarily consumed in overcoming the natural defenses of the gastrointestinal tract, and that this time relationship is retained in immune mice.

Immunization per Os.

The experiments described bring out the nature and the limitations of the immunizing process so far as it is affected by the use of killed cultures injected into the pleural or peritoneal cavity and the subcutaneous tissues. However, the parenteral introduction of the bacilli, whether living or dead, is, as it were, a circumvention of the ordinary or natural process of infection and even perhaps of immunization. Mouse typhoid, so called, is induced by the ingestion of certain paratyphoid-enteritidis strains; and it is to be supposed that in the course of an outbreak of mouse typhoid some animals ingest the cultures, resist infection, and survive. That this is not a hypothesis is shown by the observations of Amoss,² by the reported studies of Topley,⁴ as well as by our own experiments related above on infection *per os*.

The next step, therefore, in our experimental studies was to attempt immunization directly *per os*. Two general lines were pursued. Certain mice were fed fixed doses of the killed cultures and others were fed very minute amounts of living organisms, in both instances not once only, but over definite periods of time. Mice thus prepared were then tested for resistance by *per os* and intraperitoneal injection of living cultures in lethal doses. Protection was established by both methods, and in contrasting the results of the intrastomachal with the intraperitoneal injections, an attempt was made to determine the occurrence of a possible local gastrointestinal immunity.

Killed Cultures per Os.—The experiments to be described are somewhat intricate, but they are given in detail just because they bring out certain difficulties attending the systematic experimental investigation of mouse typhoid infections.

24 mice were fed daily for 30 days with bread soaked in a broth culture of *Bacillus M. T. II*, killed by heating to 55°C. for 1 hour. All the animals were

⁴ Topley, W. W. C., *J. Hyg.*, 1920–21, xix, 350; *Lancet*, 1919, ii, 1.

living at the end of the feeding period, but during the next 30 days 12 died and at autopsy yielded cultures of *Bacillus M. T. II*. This latter point will be considered presently. The 12 survivors and 12 controls were then given by stomach tube 1 cc. of a living 1:100, 16 hour broth culture of *Strain M. T. II*. Of these, 1 "vaccinated" mouse died on the 12th day and yielded at autopsy a positive culture; 7 controls died within 20 days (Table XIII). The 11 survivors were then tested for blood agglutinins and for carriage of *Bacillus M. T. II*. The blood serum of 5 mice agglutinated the bacillus completely and of 3 mice partially in dilutions of 1:200; 6 of the 11 mice proved fecal carriers of the bacillus.

TABLE XIII.

Per Os Injection of Strain M. T. II into Mice "Vaccinated" per Os with Killed Cultures.

Mice.	No. of mice.	Duration of life in fatal cases.	No. of survivors.
		days	
"Vaccinated."	12	12	11*
Controls.	12	6-7-8-9-12-17-20	5

* "Vaccinated" survivors were used again after 37 days (see Table XIV).

TABLE XIV.

Intraperitoneal Injection of Strain M. T. II into Mice "Vaccinated" per Os with Killed Cultures.

Mice.	No. of mice.	Broth dilution.	Duration of life in fatal cases.	No. of survivors.
			days	
"Vaccinated."	11	1:500		11*
Control.	1	1:100	1	0
"	5	1:500	1-3-3-4-4	0
"	1	1:1,000	3	0
"	1	1:10,000	8	0

* Survivors were discarded after 30 days.

The 11 mice were next injected intraperitoneally with 1 cc. of a 1:500 living broth culture 16 hours old. The control mice were similarly injected with dilutions of 1:100, 1:500, 1:1,000, and 1:10,000. Table XIV presents the result of these tests. While all the control animals succumbed to this active living culture, the treated animals successfully resisted many lethal doses. Not only had the 11 mice become refractory to intrastomachal injections of living cultures which at most converted them into "carriers," but they resisted intraperitoneal injections as well.

The following interpretations of this experiment are offered. The first 12 mice which succumbed to M. T. II infection may have responded to a possible very few living bacilli contained in the "vaccine" (control plates on 3 of the 30 days showed one or two colonies) or may have been infected by the attendant who, in caring for the mice, spread living bacilli from cage to cage. Although this was noted during the epidemiological experiments of Amoss,² it may be stated that in our own study great precaution was taken to avoid this complication and that numbers of other normal mice held as controls at the same time and throughout the experiments did not spontaneously develop mouse typhoid. Whether the surviving "vaccinated" mice which resisted the subsequent lethal dose *per os* were protected by the repeated and large doses of killed cultures or the very few possible live organisms which they may have ingested, cannot, from this experiment, be definitely stated; the former supposition, however, is very much more probable.

Living Cultures per Os.—Experimental data as well as observations on the manner of spread of the infection in epidemics of mouse typhoid all indicate that certain mice receive the bacilli of mouse typhoid into the stomach and intestines without succumbing to the disease itself. These facts, taken together with the effect of killed cultures fed *per os* in inducing resistance to infection by living bacilli, led to the employment of minute doses of the living cultures in order to develop this refractory state.

The procedure was as follows: A 14 hour broth culture of Strain M. T. II was fed to 18 mice, so that 3 received 1 cc. each of a 1:1,000 dilution every day, 3 received the same treatment with a 1:10,000 dilution; 3 received the 1:1,000 dilution every 2nd day, 3 the 1:10,000 dilution every 2nd day; 3 the 1:1,000 and 3 the 1:10,000 dilutions every 3rd day. Feedings were discontinued after 34 days. During this period and a subsequent 27 days, all except 7 mice had succumbed to infection with *Bacillus* M. T. II. The 7 survivors were distributed as follows: 1 from 1:10,000 every day group; 1 from 1:1,000 every 2nd day group; 1 from 1:10,000 every 2nd day group; 2 from 1:1,000 every 3rd day group; and 2 from 1:10,000 every 3rd day group. The 7 survivors, along with 7 control mice, were now given by stomach tube 1 cc. of a 1:100 dilution of Culture M. T. II. Table XV shows the result.

The 6 surviving mice were tested (a) for agglutination and (b) for carriage of *Bacillus* M. T. II. The blood serum of 1 mouse agglutinated the bacilli completely in a dilution of 1:200, and 2 of the 6 proved to be fecal carriers. They

were then injected intraperitoneally with 1 cc. of a 1:500 living broth culture 16 hours old. The control mice were similarly infected with dilutions of 1:100, 1:500, 1:1,000, and 1:10,000. Table XVI summarizes the duration of life in experimental and control animals.

TABLE XV.

Per Os Injection of Strain M. T. II into Mice "Vaccinated" per Os with Live Cultures.

Mice.	No. of mice.	Duration of life in fatal cases.	No. of survivors.
		<i>days</i>	
"Vaccinated."	7	7*	6†
Controls.	7	5-6-6-7-9-19-36	0

* This mouse showed no pathological lesions at autopsy. Heart's blood and spleen were sterile.

† Survivors were used again after 38 days (see Table XVI).

TABLE XVI.

Intraperitoneal Injection of Strain M. T. II into Mice "Vaccinated" per Os with Live Cultures.

Mice.	No. of mice.	Broth dilution.	Duration of life in fatal cases.	No. of survivors.
			<i>days</i>	
"Vaccinated."	6	1:500		6*
Control.	1	1:100	1	0
"	5	1:500	1-3-3-4-4	0
"	1	1:1,000	3	0
"	1	1:10,000	8	0

* Survivors were discarded after 30 days.

The results of this experiment are of considerable interest in that they bring out the varying capacities of mice of one age and condition to accommodate to sublethal doses of a given culture of Strain M. T. II introduced into the stomach. The response to wide fluctuations of dose is particularly informing. And the experiment shows unmistakably that the initially and doubtless reinforced refractory mice may come to resist intrastomachal or intraperitoneal doses of the living culture to which all controls succumb.

That protection is afforded certain mice by successive feedings either of heat-killed or living cultures of this paratyphoid-enteritidis strain is indicated by the above experiments. The next tests were devised to bring out the mechanism of this protection—whether, for example, it is a local process confined to the gastrointestinal tract or a general phenomenon operating against the subsequent injection of organisms introduced into the peritoneal cavity as well as into the stomach.

50 mice were tested for blood agglutinins in serum dilutions of 1:20 and 1:200. All were negative. Stool cultures of these mice showed no organisms of the paratyphoid-enteritidis group. "Vaccine" prepared as follows was then added

TABLE XVII.

Intraperitoneal and per Os Injection of Strain M. T. II into Mice "Vaccinated" per Os with Killed Cultures.

Mice.	No. of mice.	Broth dilution.	Duration of life in fatal cases.	No. of survivors.
			<i>days</i>	
Intraperitoneal controls.	4	1:100	1-2-3-3	0
“ “	1	1:1,000	8	0
“ “	1	1:10,000	9	0
“ “	1	1:100,000	7	0
“ “vaccinated.”	16	1:100	4-4-4-4-6-6-6-8-8-9-16	4*
<i>Per os</i> controls.	1	1:10	7	0
“ “ “	4	1:100	9-10-50	1
“ “ “vaccinated.”	17	1:100	5-6-7-11-12-16-17-25-31-51	7

* Survivors were discarded after 70 days.

daily to the feedings. 10 cc. of a 24 hour broth culture of Strain M. T. II, heated at 55°C. for 2 hours, were added to 40 cc. of milk, soaked up with bread, and divided roughly into ten parts so that each jar of 5 mice received approximately 1 cc. of the original "vaccine." This relatively small dose was continued over a period of 4 weeks. After an interval of 1 week, when it was seen that all the mice were in good condition, agglutination tests were again set up and stool cultures were taken, with negative results.

16 of these mice were then given intraperitoneally 1 cc. of a 1:100 dilution of a 24 hour broth culture of Strain M. T. II; 17 mice received the same dosage *per os*. Controls were injected intraperitoneally and *per os* with doses from 1:10 to 1:100,000. The duration of life in the series is analyzed in Table XVII and Text-fig. 6. Intraperitoneal injection was lethal in a dilution of 1:100,000; the intraperitoneal controls were dead in 3 days. Of the "vaccinated" mice

receiving the intraperitoneal injection, 30 per cent died on the 4th day, 75 per cent were dead on the 16th day, and 25 per cent survived over a period of 10 weeks. The animals receiving live organisms *per os* were somewhat irregular; 80 per cent of the controls succumbed, while only 55 per cent of the "vaccinated" mice died within a period of 10 weeks.

In this experiment we have established, therefore, as a response to the continued ingestion of killed organisms in small doses, a partial protection against live organisms introduced by mouth or by way of the peritoneal cavity, and this protection is in the nature of a general rather than a local immunity.

Finally, to show the general nature of the protection in a more striking manner, immunization was carried out with live organisms.

TABLE XVIII.

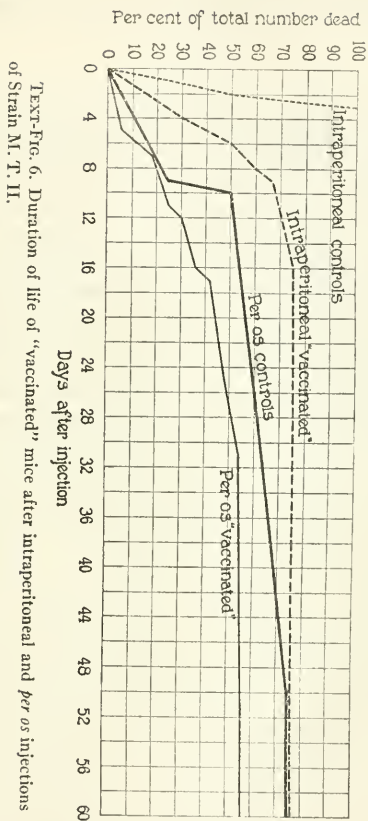
Intraperitoneal Injection of Strain M. T. II into Mice "Vaccinated" per Os with Live Cultures.

Mice.	No. of mice.	Broth dilution.	Duration of life in fatal cases.	No. of survivors.
			<i>days</i>	
Experimental.	18	1:1,000	2-6-12-16	14*
Controls.	1	1:100	1	0
"	7	1:1,000	1-1-1-2-2-4	0
"	1	1:10,000	3	0

* Survivors were discarded after 30 days.

25 mice were tested for blood agglutinins for Bacillus M. T. II with serum dilutions of 1:20 and 1:200. None of the mice reacted. Daily feedings of approximately 1 cc. per mouse were now begun with a 16 hour living broth culture of Strain M. T. II diluted 1:10,000 in milk. The total quantities were as follows: 5 mice received 5, 5 received 7, 5 received 9, 5 received 11, and 5 received 13 feedings. Within a 20 day period, 6 of the fed mice succumbed to mouse typhoid infection. The second test for agglutinins was made 13 days after the feedings began and none were found. 23 days later, or 36 days after the experiment began, of 18 surviving mice, 7 showed blood agglutinins in 1:200 dilution. Only 1 mouse was determined to be an M. T. II bacillus carrier. This experiment was terminated by inoculating intraperitoneally the entire 18 survivors of the feeding series along with controls with 1 cc. of a 1:1,000 dilution of an 8 hour living broth culture of Strain M. T. II. Table XVIII shows the result.

While all the controls died, 14 of the fed mice survived. None of the 4 fatalities had previously shown blood agglutinins. It may be



seen, therefore, that mice which ingest living cultures of Strain M. T. II and survive may develop a general immunity accompanied in a certain number of cases by blood agglutinins.

DISCUSSION.

As part of a more general study of mouse typhoid infection the experiments in this paper relate to the manner of interaction between the mouse and a native pathogen belonging to the paratyphoid-enteritidis group. The results may be stated in summary about as follows:

Among any considerable number of healthy mice of a given age and size a small number will prove refractory to inoculation with a minimum lethal dose of a mouse strain of *Bacillus pestis caviæ*, no matter into what part of the body the proper injection is made. Minimum lethal dosage is, therefore, a highly relative matter.

Taking the far greater part of the mice chosen for experiment, however, the duration of life and type of infection depend on the dose, the site of injection, and on the individual resistance of the animals as ordinarily present or as artificially produced through immunization. Lesions found at autopsy depend somewhat upon the site of the injection, but chiefly upon the duration of life after the inoculation.

When fixed doses of this organism are injected into the pleura and peritoneum of normal mice, there occurs first an initial period of lag in bacterial growth, during which time—4 to 6 hours—the number of bacilli decreases; subsequently the bacilli multiply rapidly until death occurs, usually within 8 days. This demonstration *in vivo* of bacterial lag parallels in a rough manner the careful work of Penfold,⁵ Chesney,⁶ and Graham-Smith⁷ on bacterial growth *in vitro*.

When mice are "vaccinated" with dead bacilli into the pleural or peritoneal cavity or even subcutaneously, the process of infection is altered. Live bacilli injected into the pleural and peritoneal cavities of such animals rapidly diminish in number and may apparently disappear entirely from the exudate. But usually after remaining at a low level for 2 or 3 days, they gradually begin to increase and continue a slow rise until death ensues, within about 18 days. Accord-

⁵ Penfold, W. J., *J. Hyg.*, 1914, xiv, 215.

⁶ Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.

⁷ Graham-Smith, G. S., *J. Hyg.*, 1920-21, xix, 133.

ing to degree of "vaccination" the subsequent inoculation of live cultures may produce death only after a greater time period or may fail to kill.

Paul Ehrlich was probably the first to demonstrate the possibility of immunization by the gastrointestinal route.⁸ By feeding ricin and abrin food cakes to mice, the animals not only developed a tolerance to the feeding of several lethal doses but resisted 400 lethal doses injected subcutaneously. Loeffler,⁹ Wolf,¹⁰ Yoshida,¹¹ and Brückner,¹² by feeding killed or living cultures of certain animal paratyphoid strains to mice, have established a definite immunity against the subsequent feeding in large doses of the homologous live organism. The controversy throughout this work as to whether the induced gastrointestinal immunity is local or general has been renewed by the recent work of Besredka with dysentery and paratyphoid bacilli on mice and rabbits.¹³ He offers the somewhat startling hypothesis that not only is the gastrointestinal immunity entirely of a local nature, but that any mode of vaccination is followed by only one type of immunity—local: "Tout comme l'immunité naturelle, l'immunité artificielle vis-à-vis des virus typhique et paratyphique repose sur celle de la paroi intestinale: elle est d'essence locale."

Throughout our work there has been no evidence of acquired local immunity as opposed to a condition of general immunity. The feeding of killed or living cultures of a mouse typhoid strain protects the mice against lethal doses of living bacilli injected *per os* or intraperitoneally. And when killed bacilli are injected into the pleura or peritoneum, those cavities are rendered no more resistant to the introduction of the living bacilli than when the "vaccination" is made subcutaneously. It seems, therefore, that protective principles following the introduction of killed or living bacilli into any part of the body operate against the later injection of living organisms, regardless of their portal of entry.

⁸ Ehrlich, P., *Deutsch. med. Woch.*, 1891, xvii, 976, 1218.

⁹ Loeffler, F., *Ged. schr. verstorb. Generalstabsarzt Armee* . . . v. Leuthold, 1906, i, 249.

¹⁰ Wolf, K., *Münch. med. Woch.*, 1908, lv, 270.

¹¹ Yoshida, E., *Arch. Hyg.*, 1909, lxix, 21.

¹² Brückner, G., *Z. Immunitätsforsch., Orig.*, 1910-11, viii, 439.

¹³ Besredka, A., and Basseches, S., *Ann. Inst. Pasteur*, 1918, xxxii, 193. Besredka, A., *Ann. Inst. Pasteur*, 1919, xxxiii, 301, 557, 882.

A word should be said concerning experimental infection *per os*, the normal portal of entry. Whereas earlier writers have experienced difficulty in producing the disease in this manner, we have found it possible, within certain limits, to infect regularly. These limits, however, are subject to such fluctuations that results must be interpreted with caution. In these experiments two facts must be remembered: first, that all tests were conducted in large battery jars containing either 3 or 6 mice and that this constant contact factor must have raised the per cent of mortality in controls as well as experimental animals; secondly, that only wide deviations in mortality curves were considered as significant.

SUMMARY.

If live cultures of a mouse strain of *Bacillus pestis caviæ* are injected intrapleurally or intraperitoneally into normal mice, there occurs an initial lag in the rate of bacterial multiplication lasting a few hours, followed by a rapid and continued acceleration of growth until the death of the animal.

If live cultures of this organism are given *per os* to normal mice, there occurs an incubation period of 5 to 6 days, after which the animal usually develops symptoms of disease and succumbs. A small percentage of mice, however, proves refractory to infection by this route.

If live cultures of this organism are injected intrapleurally or intraperitoneally into mice previously "vaccinated" intrapleurally or intraperitoneally, they are partially destroyed and held in check by the protective mechanisms of the animal body for 2 or 3 days. Subsequently the rate of bacterial multiplication increases gradually until the death of the animal. The partial protection following this type of "vaccination" is entirely of a general nature; no evidence of a local immunity has been obtained.

Mice given one, two, or three subcutaneous doses of "vaccine" show a similar relative increase in resistance to the subsequent intraperitoneal or *per os* injection of live organisms.

Feeding mice live or killed cultures of this organism induces a definite protection against subsequent intrastomachal and intraperitoneal injections of live organisms. The immunity developed in this way is also of a general as opposed to a local nature.

IDENTIFICATION OF A PARATYPHOID-ENTERITIDIS STRAIN ASSOCIATED WITH EPIZOOTICS OF MOUSE TYPHOID.*

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In other papers of this series, Amoss¹ and Lynch² have described epizootics of mouse typhoid associated with a bacillus of the paratyphoid-enteritidis group, and with this same organism, designated as Strain M. T. II, the author has studied certain corollary protection and immunity phenomena.³ It seems important, therefore, to classify definitely this M. T. II strain and to relate it as far as possible to other similar pathogens.

B. typhi murium, isolated by Loeffler⁴ from an epizootic of laboratory mice and related to the colon-typhoid group, was considered by him to be the causative microbe of mouse typhoid. In France this etiological rôle was assigned to a similar organism, Danysz' bacillus, recovered from a plague of field mice.⁵ Later serological studies of Bainbridge⁶ on the paratyphoid and food poisoning bacilli indicate that Danysz' bacillus is identical with *B. enteritidis* Gärtner and that *B. typhi murium* does not exist as an entity since some strains correspond to

* In a communication to the Society for Experimental Biology and Medicine (Webster, L. T., *Proc. Soc. Exp. Biol. and Med.*, 1921-22, xix, 71) this organism was designated as *B. enteritidis (murium)*.

¹ Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 25.

² Lynch, C. J., *J. Exp. Med.*, 1922, xxxvi, 15.

³ Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 71.

⁴ Loeffler, F., *Centr. Bakt.*, 1892, xi, 129.

⁵ Danysz, J., *Ann. Inst. Pasteur*, 1900, xiv, 193.

⁶ Bainbridge, F. A., *J. Path. and Bact.*, 1909, xiii, 443.

B. enteritidis and others to *B. aertrycke*.⁷ Krumwiede⁸ has found that strains of so called *B. typhi murium* belong either to the enteritidis group or to a class of paratyphoid-like strains of rodent origin (*B. pestis caviae*).⁹ Finally, throughout Topley's study of an experimental mouse epizootic induced by the feeding of *B. enteritidis*, his autopsy protocols note the recovery of *B. enteritidis* and *B. aertrycke*.^{7:10} Apparently, then, *B. typhi murium* has no significance as an entity; Danysz' bacillus is synonymous with *B. enteritidis* Gärtner; and the microbes usually associated with mouse typhoid are *B. enteritidis* Gärtner, *B. aertrycke*, and *B. pestis caviae* Smith.

Morphological and Cultural Characteristics.

Strain M. T. II is a Gram-negative, motile, non-sporulating bacillus. Agar colonies are thin, bluish, and somewhat translucent, with irregular edges. Dextrose, levulose, maltose, mannitol, xylose, arabinose, rhamnose, and inosite are fermented. Gas is formed, milk is not coagulated, indole is not produced, lead acetate medium is blackened.

These characteristics place the organism in the paratyphoid-enteritidis group. The fermentation of arabinose and the reaction in lead acetate medium would tend to exclude *Bacillus suispestifer*, while the vigorous production of acid in inosite eliminates *Bacillus enteritidis* and favors the paratyphoid group. However, it is necessary to correlate these observations with the more accurate information derived from serological tests.

Serological Studies.

An immune rabbit serum was prepared from Strain M. T. II. Stock cultures of the paratyphoid-enteritidis group were seeded into plain broth, pH 7.4, incubated for 24 hours, and mixed with equal parts of 0.2 per cent formalin in 0.85 per cent salt solution. Agglutination tubes were set up to contain one part of serum dilution to nine

⁷ Topley, Weir, and Wilson (Topley, W. W. C., Weir, H. B., and Wilson, G. S., *J. Hyg.*, 1921, xx, 241) make the following statement in regard to paratyphoid bacilli of animal origin: "It is probable that *B. aertrycke* belongs to this subgroup, and also those *B. suispestifer* strains of German origin which were studied by Bainbridge and O'Brien (1911). The bacilli isolated from mice dying during our own experiments, and hitherto referred to as *B. suispestifer*, have all the characteristics of this subgroup and should be placed in it."

⁸ Krumwiede, C., Jr., Valentine, E., and Kohn, L. A., *J. Med. Research*, 1918-19, xxxix, 449.

⁹ Wherry, W. B., *J. Infect. Dis.*, 1908, v, 519.

¹⁰ Topley, W. W. C., *J. Hyg.*, 1920-21, xix, 350.

parts of formalinized antigen. The several strains employed may be described as follows:

M. T. II. Unknown strain for identification.

B. pestis caviæ, No. 146. (Ferry 63).⁸

320 mutton type. "Aertrycke" strain from Schütze;¹¹ called by him "Mutton type Calf 6."

B. choleraë suis, No. 350 (Smith). Isolated by TenBroeck in 1919.

B. paratyphosus B. No. 178 Army strain.

313 Stanley. "Aertrycke" strain from Schütze; Hutchins.¹¹

315 G. "Aertrycke" strain from Schütze; monkey.¹¹

316 Reading. "Aertrycke" strain from Schütze; water supply.¹¹

317 Newport. "Aertrycke" strain from Schütze; Fentry strain; isolated by Perry.¹¹

B. enteritidis, No. 47. Gärtner.

B. enteritidis, No. 273. McWeeny strain; from Jordan.

B. abortus equi, 215 Meyer No. 7. Kentucky.

B. paratyphosus A. Army strain.

From Table I it may be seen that while Serum M. T. II fails to agglutinate *Bacillus enteritidis*, *Bacillus abortus equi*, and *Bacillus paratyphosus A*, and while it reacts only slightly with the Army strain of *Bacillus paratyphosus B*, *Bacillus choleraë suis*, and the Stanley, G, Reading, and Newport *aertrycke* strains of Schütze, it does agglutinate the "mutton" strain of Schütze and *Bacillus pestis caviæ* to a titer approximating that of the homologous strain.

To emphasize this relationship, sera of the various type strains were set up against Strain M. T. II (Table II).

This cross-agglutination eliminates the Army Para B, *Bacillus choleraë suis*, and the Stanley, G, Reading, and Newport strains. "Mutton" serum, however, agglutinates Strain M. T. II to nearly as high a titer as the homologous strain and *Bacillus pestis caviæ* serum agglutinates Strain M. T. II and homologous strain to equal titer.

Final evidence relating Strain M. T. II to *Bacillus pestis caviæ* and the "mutton" strain was obtained by absorption tests.

Some quantitative measurement of absorbing antigen is necessary for accurate interpretation of absorption phenomena. In these tests,

¹¹ Schütze, H., *Lancet*, 1920, i, 93.

therefore, a constant ratio between each serum volume and the volume of the absorbing strain was determined by measuring the volume of packed cell antigen and then adding the calculated amount of serum diluted 1:10. For the low titer "mutton" serum this ratio was 1:4; for the *Bacillus pestis caviæ* serum a 1:2 ratio was employed, while in the case of Serum M. T. II, it was necessary to absorb twice with a ratio of packed cells to serum of 1:3. For controls each absorption

TABLE I.
Agglutination with Serum M. T. II against Various Strains.

Strain.	Serum M. T. II dilution.							
	1:100	1:1,000	1:5,000	1:7,000	1:9,000	1:10,000	1:20,000	1:50,000
M. T. II.	++	++	++	++	++	++	++	—
<i>B. pestis caviæ</i> , No. 146.	++	+1	+1	+1	+1	+1	+	—
320 mutton.	++	++	++	+1	+1	+1	+	—
<i>B. cholerae suis</i> , No. 350 (Smith).	+1	+1	—	—	—	—	—	—
Para B Army.	+1	+1	—	—	—	—	—	—
313 Stanley.	+1	+	—	—	—	—	—	—
315 G.	+1	+1	—	—	—	—	—	—
316 Reading.	+1	+1	—	—	—	—	—	—
317 Newport.	+	+	—	—	—	—	—	—
<i>B. enteritidis</i> , No. 47.	—	—	—	—	—	—	—	—
" " " 273.	—	—	—	—	—	—	—	—
" <i>abortus equi</i> .	—	—	—	—	—	—	—	—
Para A Army.	—	—	—	—	—	—	—	—

++ indicates complete agglutination; +1, marked agglutination; +, slight agglutination; 1, trace of agglutination.

test was paralleled by agglutination of unabsorbed serum with homologous and heterologous strains and by absorption and subsequent agglutination with the homologous strain. The results are presented in Tables III to VI.

Table III shows that Strain M. T. II completely absorbed its own agglutinins from "mutton" serum and 98 per cent of the homologous agglutinins. Conversely (Table IV), the "mutton" strain, after one absorption, removed 90 per cent of its own agglutinins from Serum M. T. II and 75 per cent of the homologous agglutinins, and, after

two absorptions, the "mutton" strain removed all of its own agglutinins and 93 per cent of the homologous agglutinins.

It is probable then, that Strain M. T. II and the "mutton" strain are antigenically very similar but not quite identical.

TABLE II.

Direct Agglutination with Various Sera against Strain M. T. II.

Serum.	Strain.	Serum dilution.											
		1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:102,400	Control.
<i>B. pestis caviæ.</i>	<i>B. pestis caviæ.</i> M. T. II.	++	++	++	++	++	++	+	1	—	—	—	—
		++	++	++	++	++	++	+	1	—	—	—	—
"Mutton."	"Mutton." M. T. II.	++	++	++	++	++	+1	1	1	—	—	—	—
		++	++	++	++	++	1	—	—	—	—	—	—
<i>B. choleraë suis</i> , No. 350.	<i>B. choleraë suis</i> , No. 350. M. T. II.	++	++	++	++	++	++	+1	+	+	1	—	—
		—	—	—	—	—	—	—	—	—	—	—	—
<i>B. paratyphosus</i> B, No. 178.	<i>B. paratyphosus</i> B. M. T. II.	++	++	++	++	++	++	+1	+	+	1	—	—
		—	—	—	—	—	—	—	—	—	—	—	—
313 Stanley.	313 Stanley. M. T. II.	++	++	++	++	++	+	1	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	—	—
315 G.	315 G. M. T. II.	++	++	++	++	++	++	+	+	1	—	—	—
		—	—	—	—	—	—	—	—	—	—	—	—
316 Reading.	316 Reading. M. T. II.	++	++	++	++	++	++	+	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	—	—
317 Newport.	317 Newport. M. T. II.	++	++	++	++	++	+	—	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	—	—

Table V shows that Strain M. T. II absorbed 97 per cent of its own agglutinins from *Bacillus pestis caviæ* serum and 97 per cent of the homologous agglutinins. Conversely (Table VI), the *Bacillus pestis caviæ* strain after one absorption removed 98 per cent of its own agglutinins from Serum M. T. II and 98 per cent of the ho-

TABLE II.

"Mutton" Serum.

[illegible]

TABLE IV.
Serum M. T. II.

[illegible]

TABLE V.
B. pestis cavie Serum.

Strain.	Unabsorbed serum.												Serum absorbed by <i>B. pestis cavie.</i>												Serum absorbed by Strain M. T. II.											
	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	Control.	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200					
<i>B. pestis cavie.</i> ...	++	++	++	++	++	++	++	++	++	++	—	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	—				
M. T. II.	++	++	++	++	++	++	++	++	++	++	—	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	—				

TABLE VI.
Serum M. T. II.

Strain.	Unabsorbed serum.										Single absorption by Strain M. T. II.										Single absorption by <i>B. pestis canis</i> .																
	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	Control.	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:103,400	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:102,400			
M. T. II.....	++	++	++	++	++	++	++	++	++	—	++	++	++	++	++	—	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++			
<i>B. pestis...</i>	++	++	++	++	++	++	++	++	++	—	++	++	++	++	++	—	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++			
<i>canis</i>	++	++	++	++	++	++	++	++	++	—	++	++	++	++	++	—	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++			
Strain.	Double absorption by Strain M. T. II.										Double absorption by <i>B. pestis canis</i> .																										
	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200				
M. T. II.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
<i>B. pestis canis</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
Strain.	Double absorption by Strain M. T. II.										Double absorption by <i>B. pestis canis</i> .																										
	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200				
M. T. II.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
<i>B. pestis canis</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				

mologous agglutinins, and, after two absorptions, the *Bacillus pestis caviæ* strain removed all of its own agglutinins and all of the homologous agglutinins from Serum M. T. II.

Clearly, then, Strain M. T. II and the type strain *Bacillus pestis caviæ* are antigenically identical.

DISCUSSION.

The "mutton" group forms the chief division in Schütze's serological study of the paratyphoid *aertrycke* types.¹¹ He says: "The Mutton group claims over 50 per cent of the strains, and would appear to be the most important; it includes strains from various countries and from animal as well as human epidemics."

The *Bacillus pestis caviæ* group, "a distinct paratyphoid type or group of bacilli, is encountered therefore in spontaneous infections in laboratory animals, especially rodents" according to Krumwiede.⁸ Thirteen guinea pigs, two mice, one cat, and one rabbit strains were found by him to be antigenically similar.

Strain M. T. II is apparently similar to the type "mutton" strain of Schütze and is identical with the type *Bacillus pestis caviæ* strain.¹²

To have closely related this mouse typhoid strain to other paratyphoid types is to emphasize once again the ubiquity of the paratyphoid group and the possibility that the various strains found in mice, rats, guinea pigs, sheep, and doubtless in other domestic animals, active, capable of producing epizootics, may likewise be human pathogens with greater or less degree of virulence. Precise information concerning the underlying principles of mouse typhoid infection and epizootics should, therefore, be of great service in the interpretation of similar phenomena of man.

CONCLUSIONS.

A bacillus of the paratyphoid-enteritidis group associated with epizootics among laboratory mice has been identified with *Bacillus pestis caviæ* Smith which produces similar affections in guinea pigs and has been very closely related to the type "mutton" *aertrycke* strain of Schütze.

¹² Unpublished studies of Krumwiede and Cooper have already demonstrated a close relation between the *B. pestis caviæ* group and the "mutton" types of Schütze.

This identification is based upon the cultural reactions of the organism, direct and cross-agglutinations, and similar absorptive capacities of the unknown and type strains.

The author wishes to thank Dr. Charles Krumwiede and Miss Georgia Cooper for the type strains and type antisera and for assistance especially as regards the technical details of the absorption method which they employ.

IMMUNOLOGICAL DISTINCTIONS OF TWO STRAINS OF THE MOUSE TYPHOID GROUP ISOLATED DURING TWO SPONTANEOUS OUTBREAKS AMONG THE SAME STOCK.

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In the cancer breeding station maintained at The Rockefeller Institute there occurred, during $2\frac{1}{2}$ years, two separate outbreaks of mouse typhoid among the 2,500 to 4,000 mice. The first of these appeared in the autumn of 1918 and the second exactly 2 years later. They are described by Lynch¹ in a separate paper of this series.

From the spleens of mice dying in the first outbreak there were isolated several strains which belonged to the paratyphoid-enteritidis group. One of them, Mouse Typhoid I, was selected for use in inciting an experimental epidemic, but before starting the experiments, this strain was passed through four mice *per os* in order to assure its pathogenic power. The strain, recovered from the fourth mouse, was used in a series of artificially produced epidemics among mice for more than 2 years. Meanwhile, antiserum had been prepared against Mouse Typhoid I, and while active in high dilution against the homologous strain, it did not agglutinate the unidentified strain. It was evident that the two strains, both belonging to the paratyphoid-enteritidis group as determined by fermentation tests, were not identical. Antiserum against the new strain was prepared, but it did not agglutinate Mouse Typhoid I. This serum, however, did agglutinate the strains isolated from the spleens of mice dying in the experimental epidemic, and the inference is that the unidentified strain (now called Mouse Typhoid II) was the organism concerned in the epidemic, for no members of Mouse Typhoid I type were obtained.

In the preliminary feeding experiment to which reference has been made, Mouse Typhoid I was employed, but Mouse Typhoid II was recovered from the spleen of the fourth mouse in the series. The

¹ Lynch, C. J., *J. Exp. Med.*, 1922, xxxvi, 15.

probable explanation is that one of the mice used in the experiment was already infected with Mouse Typhoid II. This view is strengthened by the fact that occasionally among the stock from which these mice were taken deaths occurred and from the spleens only Mouse Typhoid II was isolated. Moreover, in the second epidemic among the cancer mice occurring in the autumn of 1920, Mouse Typhoid II was regularly obtained.

EXPERIMENTAL.

Agglutination Tests.

Monovalent sera were prepared by immunizing rabbits against Mouse Typhoid I, Mouse Typhoid II, and Paratyphoid B Rowland.²

TABLE I.

Agglutination of Various Strains of the Paratyphoid-Enteritidis Group in Three Monovalent Antisera.

Antigen.	Highest dilution showing complete agglutination.		
	Serum.		
	Mouse Typhoid I.	Mouse Typhoid II.	Paratyphoid B Rowland.
Mouse Typhoid I.	1:2,000	0	0
“ “ II.	0	1:1,500	1:800
Paratyphoid B Rowland (Army Medical School).....	0	1:800	1:1,200
Paratyphoid B Black (Army Medical School). ..	0	1:1,000	1:1,200
“ “ Kendall I.	0	1:1,000	1:4,000
“ “ “ II.	0	1:1,000	1:5,000
Enteritidis—Kendall.	1:2,000	0	0
“ 18 (American Museum of Natural History).....	0	0	0
Enteritidis 273 Gärtner.....	1:800	0	0
Animal typhoid—Smith.			
Mouse 2. }	>1:400*	0	0
Rat. }			
Guinea pig. }			
Dog 1. }			
Calif.	0	>1:400*	0

* Higher dilution not made.

² Paratyphoid B Rowland is the strain used by the Army Medical School in the preparation of the triple typhoid vaccine.

The results of the agglutination tests with these strains and enteritidis strains and other paratyphoid B strains³ are shown in Table I.

It is evident that Mouse Typhoid II is closely allied antigenically to the human paratyphoid B strains tested, while Mouse Typhoid I is totally different. Mouse Typhoid I is related to the enteritidis strains Kendall and Gärtner 273 but not to the culture obtained from the American Museum of Natural History.

Animal Strains.

The following animal typhoid strains⁴ were roughly classified by agglutination in Mouse Typhoid I and Mouse Typhoid II serum in a dilution of 1:400, as follows: Mouse 2, rat, guinea pig, and Dog 1 strains were completely agglutinated by Mouse Typhoid I serum in 1:400 and not by Mouse Typhoid II serum; while the calf strain was completely agglutinated by Mouse Typhoid II serum and not by Mouse Typhoid I serum.

Absorption Tests.

Mouse Typhoid II and Para B Sera.

Antigen for absorption was prepared by washing 16 hour growths from slant agar pH 7.4 with isotonic salt solution, filtering through cotton wool, and standardizing in a turbidimeter. The absorptions with the same amount of antigen in every case were made four times during 3 hours at 37°C. in dilutions 1:10, 1:20, 1:25, and 1:50 respectively. The results of agglutination tests on the Mouse Typhoid II and Para B sera after absorption are given in Table II.

It appears from these tables that Mouse Typhoid II and Para B are not identical. Para B Rowland and Black strains absorb agglutinins against themselves from Mouse Typhoid II serum, but leave some Mouse Typhoid II agglutinins. Para B serum is absorbed more slowly even by the homologous strain. The difference between Mouse Typhoid II and Para B Rowland and Black is also

³ Strains of paratyphoid B and enteritidis were obtained from the Army Medical School, Dr. A. I. Kendall, and the American Museum of Natural History.

⁴ These strains were furnished by Dr. Theobald Smith.

TABLE II.
Agglutination after Absorption.

Test strain.	Before absorption.	Absorbing Strain.					
		Mouse Typhoid II.	Para B Rowland.	Para B Black.	Para B Kendall I.*	Para B Kendall II.*	Mouse Typhoid I.

Mouse Typhoid II serum.							
Mouse Typhoid II.....	1:1,500	0	++1:400	++1:500	+1:200 ++1:100	+1:300 ++1:200	++1:1,500
Para B Rowland.....	1:800	0	0	0	+1:200 ++1:100	0	—
“ “ Black.....	1:1,000	0	0	0	+1:200 ++1:100	0	—
“ “ Kendall I.....	1:1,000	++1:100	++1:200	++1:100, 1:200	++1:400 ++1:300	++1:400 ++1:300	—
“ “ “ II.....	1:1,000	++1:100	++1:200	++1:100	++1:400 ++1:300	++1:200	—
Calf typhoid.....	1:400†	0	—	—	—	—	—

Para B Rowland serum. Regular technique four times in 3 hrs.							
Mouse Typhoid II.....	1:800	+1:100	+1:100	+1:100	++1:500	++1:500	—
Para B Rowland.....	1:1,200	++1:400 ++1:100	+1:300 +1:100	++1:100	++1:500	++1:800	—
“ “ Black.....	1:1,200	++1:300 ++1:100	++1:300 ++1:100	++1:200	++1:500	++1:800	—
“ “ Kendall I.....	1:4,000	++1:1,000	++1:500	++1:100	++1:1,000 ++1:2,000	++1:2,000	—
“ “ “ II.....	1:5,000	++1:2,000	++1:800	++1:500	++1:2,000 ++1:1,000	++1:2,000	—

shown after absorption eight times over 16 hours at 37°C., when Mouse Typhoid II removes all agglutinins reacting against it but leaves agglutinins for Para B strains.

Para B Kendall I and II are still slower in absorbing and are not identical with Para B Rowland.

Mouse Typhoid I has no power of absorption in Mouse Typhoid II serum.

Mouse Typhoid I Serum.

By direct agglutination Mouse Typhoid I seems to be related to four animal typhoid strains and also to Enteritidis Kendall and

TABLE III.
Agglutination after Absorption.

Test strain.	Before absorption.	Absorbing strains.			
		Mouse Typhoid I.	Enteritidis 18.	Enteritidis Kendall.	Enteritidis 273.
Mouse Typhoid I.....	1:2,000	0	+1:1,000 +++1:500	+1:800 +++1:400	+1:1,000 +++1:200
Enteritidis 18.....	0	0	0	0	—
“ Kendall.....	1:2,000	0	+1:1,000 +++1:400	0	—
“ 273.....	1:800	0	—	—	0
Mouse Typhoid 2 (Smith) ..	>1:400	0	—	—	0
Rat typhoid (Smith).....	>1:400	0	—	—	0
Guinea pig typhoid (Smith)	>1:400	0	—	—	+1:300
Dog Typhoid 1 (Smith)....	>1:400	0	—	—	+1:800

Enteritidis 273 Gärtner, but the latter are differentiated by absorption with the method already described.

Table III shows that Mouse Typhoid I removes all agglutinins from Mouse Typhoid I serum, but Enteritidis Kendall and 273 absorb all agglutinins against themselves from Mouse Typhoid I serum, but not all which are active against Mouse Typhoid I. The fact that Enteritidis 18, though not agglutinated in Mouse Typhoid I serum, apparently absorbs some of the agglutinins, is perhaps an example of non-specific or physical adsorption.

SUMMARY.

Two strains of the paratyphoid B-enteritidis group causing separate epidemics of mouse typhoid among 2,500 to 4,000 cancer breeding mice are found to be antigenically different. Mouse Typhoid I, isolated from the first outbreak, is related but not identical with two strains of enteritidis, while Mouse Typhoid II is related to but not identical with the human paratyphoid B strains.

In a separate paper in this series, Webster⁵ has identified Mouse Typhoid II strain with *Bacillus pestis caviae* Smith and has suggested its close relation to the *Bacillus aertrycke* (mutton) group of Schütze.

⁵ Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 97.

SURFACE TENSION OF SERUM.

III. RECOVERY AFTER LOWERING BY SURFACE-ACTIVE SUBSTANCES.

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PLATE 2.

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I.

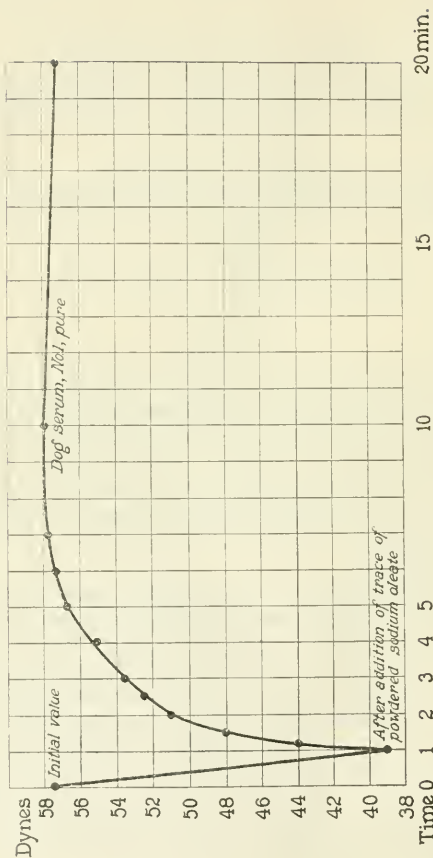
INTRODUCTION.

It has been shown in previous papers^{1,2} that surface tension of serum and of solutions of serum varies in function of the time, and that the lowering of the surface tension seems to follow the laws of adsorption in the surface layer. The assumption was made that some of the constituents of the serum having a strong action upon the surface tension of water were actually adsorbed in function of time, or that certain changes in the arrangements of the group molecules took place in the surface layer.

Comparative experiments made with certain surface-active organic compounds, such as sodium oleate, glycocholate, and saponin, showed similar results.² But when sodium oleate, for instance, was added to serum or to a solution of serum, a converse phenomenon happened. A very considerable drop occurred first, immediately followed by a rise, which was function of the dilution of the serum and, in the case of pure serum, raised the surface tension to its normal value in a few minutes (Text-fig. 1). In other words, the action of sodium oleate was immediately counteracted and, in certain cases, entirely inhibited by an antagonistic or buffing action due to certain constituents of the serum. This explains why in jaundice large quantities of sodium

¹ du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 575.

² du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 707.



TEXT-FIG. 1. Rise of surface tension of pure serum after a drop due to the addition of sodium oleate.

glycocholate and taurocholate are present in the blood without producing any hemolysis. The same amount dissolved in saline solution would bring forth a strong lowering of surface tension and would hemolyze red cells powerfully. The purpose of this paper is the study of this phenomenon.

II.

EXPERIMENTAL.

The technique used was described in detail in the previous papers.^{1,2} It will suffice to say that the measurements of the surface tension of the same layer of liquid were made by means of the du Noüy tensiometer.

TABLE I.

Rise of Surface Tension of Serum in Function of Time after a Drop Due to the Addition of Sodium Oleate.

Pure Dog Serum, No. 1 (Text-Fig. 1).

Temperature 22°C.

About 1/10,000 in weight of powdered sodium oleate was used.

Time.	Surface tension.
	<i>dynes</i>
Before addition of sodium oleate.....	57.5
After " " " "	39.0
" 15 sec.	44.0
" 30 "	48.0
" 1 min.	51.0
" 1.5 "	52.5
" 2 "	53.5
" 3 "	55.0
" 4 "	56.8
" 5 "	57.3
" 6 "	57.6
" 9 "	58.0
" 20 "	57.6

eter. As usual, the utmost precautions were taken as to the cleanliness of the glassware (watch-glasses, test-tubes, pipettes, stirring rods), which was washed in soda solution, then boiled for 30 minutes in a sulfuric acid and sodium dichromate cleaning fluid. Fresh dog and rabbit sera were used. When no graduated pipettes were needed, the

pipettes were chosen with very nearly the same capacity, measured by pressing evenly on the rubber nipple (1.8 to 1.9 cc.).

Immediately after the first measurement, a certain amount of sodium oleate was added and the surface tension measured. From 30 to 60 seconds elapsed between the two measurements. Although sodium glycocholate and taurocholate act in the same way, sodium oleate was chosen on account of its more marked action on surface tension. These measurements were made at regular intervals, generally 1 minute, at the beginning; in certain cases of very rapid recovery (pure serum), they were made at intervals of 15 seconds

TABLE II.

Rise of Surface Tension of Serum in Function of Time after a Drop Due to the Addition of Sodium Oleate.

Pure Dog Serum, No. 2 (Text-Fig. 2).

Temperature 22°C.

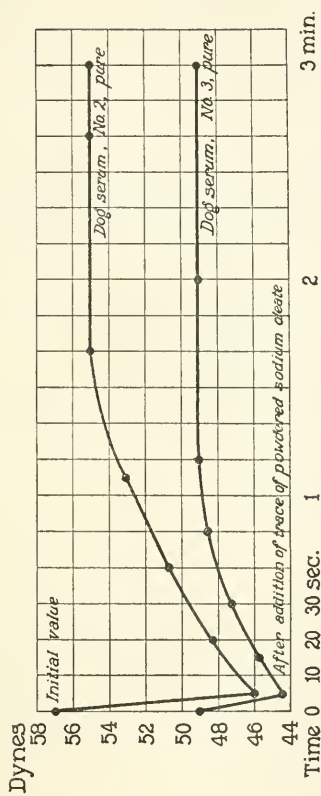
About 1/10,000 in weight of powdered sodium oleate was used.

Time.	Surface tension.
	<i>dynes</i>
Before addition of sodium oleate.....	56.0
30 sec. after addition of sodium oleate.....	46.0
After 1 min.....	53.0
“ 1.5 “	55.0
“ 2.5 “	55.0

during the first 1 or 2 minutes. In order to avoid experimental errors, all the measurements were repeated, in certain cases four times.

1. *Pure Serum.*—The results obtained with Serum 1 are shown in Table I. This serum had previously been heated at 56°C. for 2 hours and did not manifest any sign of spontaneous lowering of surface tension when exposed in a watch-glass. After 9 minutes the value of the surface tension was higher than the initial value. An attempt to explain this fact will be made in a later paragraph. With Serum 2 (Table II and Text-fig. 2) the final surface tension was 1 dyne lower than the initial value.

With Sera 1, 2, and 3 (Tables I to III), the rise was extremely rapid, and after a drop of 18.5 dynes, the initial value was reached in 5



TEXT-FIG. 2. Rise of surface tension of pure serum after a drop due to the addition of sodium oleate.

minutes in the first experiment; in the second, the drop being much smaller (10 dynes), the initial value was reached in $1\frac{1}{2}$ minutes, and in the third case (4.5 dynes), in 80 seconds. The same amount of sodium oleate added to saline solution brought its surface tension down to about 32.5 dynes permanently. Every serum seemed to react in its own specific way as far as time and final value were concerned.

The result obtained by the addition of 1 drop of sodium oleate solution at a concentration of 1/100 is given in Table IV and Text-fig. 3. The final concentration of sodium oleate in the solution was about 1/12,000. The concentration of sodium oleate was much smaller and

TABLE III.

Rise of Surface Tension of Serum in Function of Time after a Drop Due to the Addition of Sodium Oleate.

Pure Dog Serum, No. 3 (Text-Fig. 2).

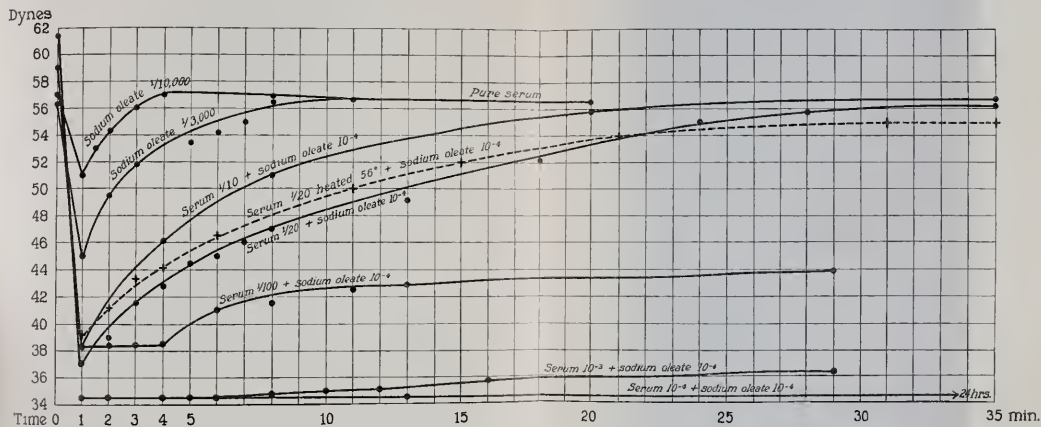
Temperature 22°C.

About 1/10,000 in weight of powdered sodium oleate was used.

Time.	Surface tension.
	<i>dynes</i>
Before addition of sodium oleate.....	49.0
30 sec. after addition of sodium oleate.....	44.5
After 40 sec.....	47.2
" 1 min.....	48.5
" 1.3 "	49.0

the drop in surface tension was reduced accordingly. In Table V the same experiment was made, but 3 drops of the same sodium oleate solution were added instead of 1, making the concentration of the surface-active substance about 1/4,000. The final surface tension is equal to the initial value. It sometimes happens that after reaching its initial value rapidly, a drop occurs, due to the normal phenomenon studied in the preceding papers.^{1,2}

2. *Dilutions of Serum.*—Dilutions of serum in saline solution at 0.9 per cent were prepared at concentrations of 1/10, 1/20, 1/100, 1/1,000, and 1/10,000. The same amount of sodium oleate was added to the same amount of solution. For these measurements, a 2 cc. graduated pipette was used. The average weight of a drop



TEXT-FIG. 3. Rise of surface tension of pure serum and serum solutions after a drop due to the addition of sodium oleate.

was 0.019 gm. The amount of serum solution was equal to 1.9 gm. The final concentration of sodium oleate equalled 1/10,000 (Table VI).

TABLE IV.

Rise of Surface Tension of Serum in Function of Time after a Drop Due to the Addition of Sodium Oleate.

Pure Rabbit Serum, No. 119 (Text-Fig. 3).

Temperature 22°C.

1 drop of sodium oleate solution, 1/100, was used.

Time.	Surface tension.
	<i>dynes</i>
Before addition of sodium oleate.....	57.0
After " " " "	51.0
" 1 min.....	54.3
" 2 "	56.0
" 3 "	57.0

TABLE V.

Rise of Surface Tension of Serum in Function of Time after a Drop Due to the Addition of Sodium Oleate.

Pure Rabbit Serum, No. 119 (Text-Fig. 3).

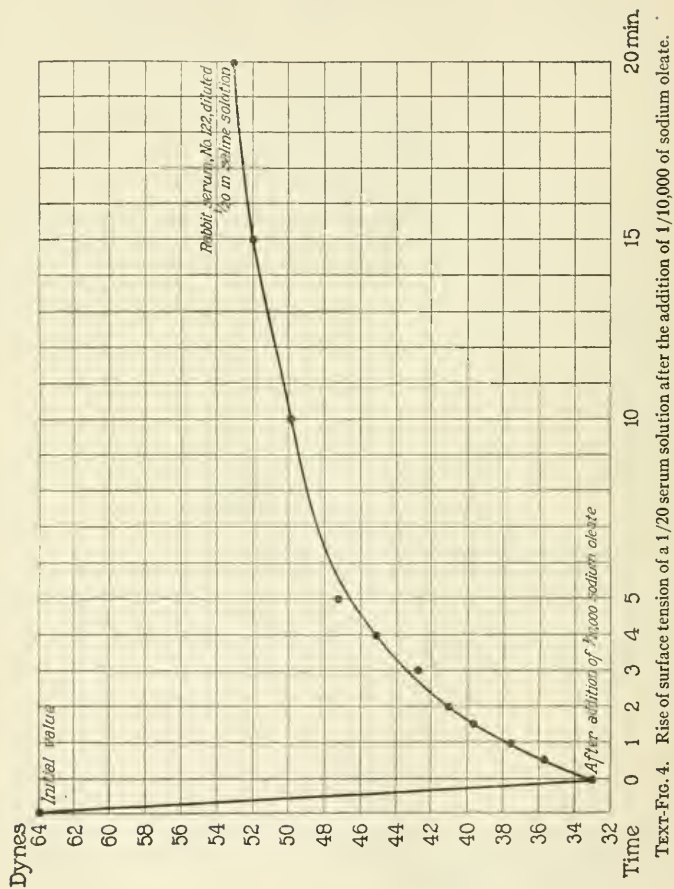
Temperature 22°C.

3 drops of sodium oleate solution, 1/100, were used.

Time.	Surface tension.
	<i>dynes</i>
Before addition of sodium oleate.....	56.3
After " " " "	45.4
" 1 min.....	49.5
" 2 "	51.8
" 4 "	53.5
" 5 "	54.2
" 6 "	55.0
" 7 "	56.5
" 27 "	56.3

The recovery of a serum solution at 1/20 (rabbit serum, No. 122) is given also in Text-fig. 4.

These figures show that at a dilution of 1/10,000 the antagonistic power of the serum was inhibited. At the end of 24 hours, the



TEXT-FIG. 4. Rise of surface tension of a 1/20 serum solution after the addition of 1/10,000 of sodium oleate.

reading was still 35.6. This phenomenon is therefore quite different from that of the decrease in surface tension of serum solutions, which showed a maximum activity in most cases at a dilution of about 1/10,000.

The results in percentage rise and in absolute rise in 30 minutes are expressed by Text-figs. 5 and 6. For the same dilutions, the time

TABLE VI.

Rise of Surface Tension of Serum Solutions in Function of Time after a Drop Due to the Addition of Sodium Oleate.

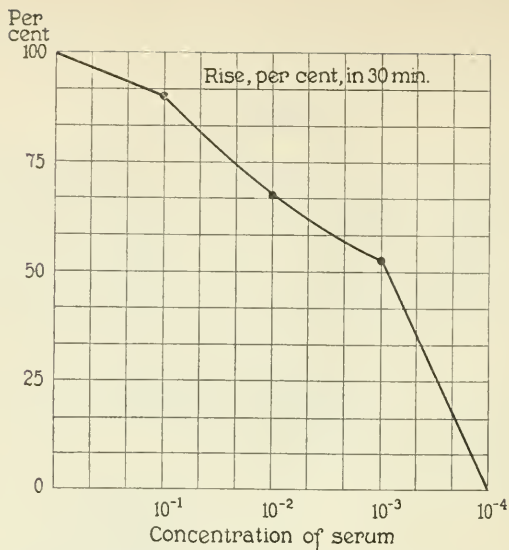
Rabbit Serum, No. 121, Diluted in Isotonic NaCl Solution (Text-Fig. 3).

Temperature 22°C.

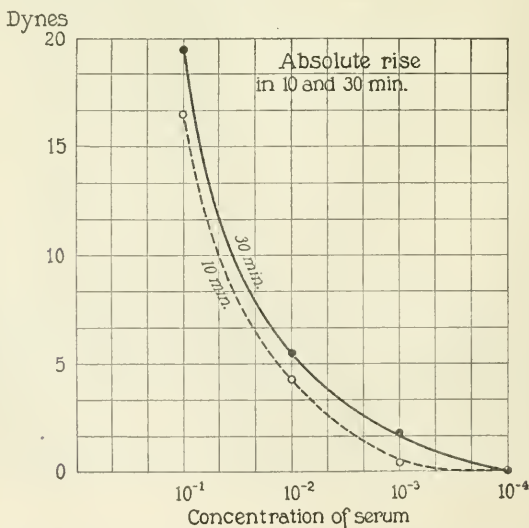
Sodium oleate, 10^{-4} , was used.

Time.	Surface tension.				
	Concentration of serum.				
	1/10	1/20	1/100	1/1,000	1/10,000
	<i>dynes</i>	<i>dynes</i>	<i>dynes</i>	<i>dynes</i>	<i>dynes</i>
Before addition of sodium oleate.....	62.5	62.0	64.0	69.5	72.0
After " " " " "	38.5	37.0	38.4	34.5	35.6
" 1 min.....		39.0	38.5	34.5	35.6
" 2 "	46.0	41.5	38.5	34.5	35.6
" 3 "		42.8		34.5	35.6
" 4 "	48.5	44.4	40.0	34.6	35.6
" 5 "		45.0	41.0	34.7	
" 6 "	51.0	46.0	41.7	34.8	
" 7 "		47.0			35.6
" 8 "	55.9			35.0	
" 9 "			42.6	35.2	
" 15 "	55.0	51.0	43.3	35.7	35.6
" 30 "	56.5	56.0	44.0	36.5	35.6

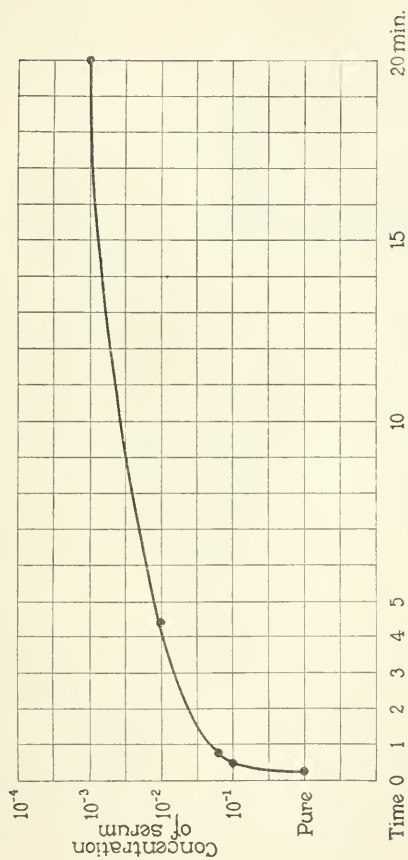
necessary to raise the surface tension of the solutions by 2 dynes is represented by Text-fig. 7. It will be seen that, by diluting the serum tenfold, the time is only doubled, while it is increased seventeenfold by diluting the serum solution again ten times; that is, to 1/100. Doubling the dilution at a concentration of 1/10, *viz.* 1/20, has the same effect as diluting pure serum tenfold. These phenomena, although difficult to interpret at present, are very probably due to the fact that surface tension is governed by the best possible arrange-



TEXT-FIG. 5. Percentage rise of surface tension of serum solutions and serum in function of the concentration of the serum after the addition of the same amount of sodium oleate. Time 30 minutes.



TEXT-FIG. 6. Rise of surface tension of serum solutions in function of the concentration of serum after the addition of the same amount of sodium oleate. Time 30 minutes.



TEXT-FIG. 7. Time necessary to raise the surface tension of serum solutions by 2 dynes after the addition of the same amount of sodium oleate.

ment of group molecules in the surface layer, as was stated in previous papers. The explanation of the broken aspect of the curve in Text-fig. 5 lies in the better knowledge of the structure of the

TABLE VII.

Quantitative Determination of the Antagonistic Action in Function of Time of a 1/10 Serum Solution.

Rabbit Serum, No. 120 (Text-Fig. 8).

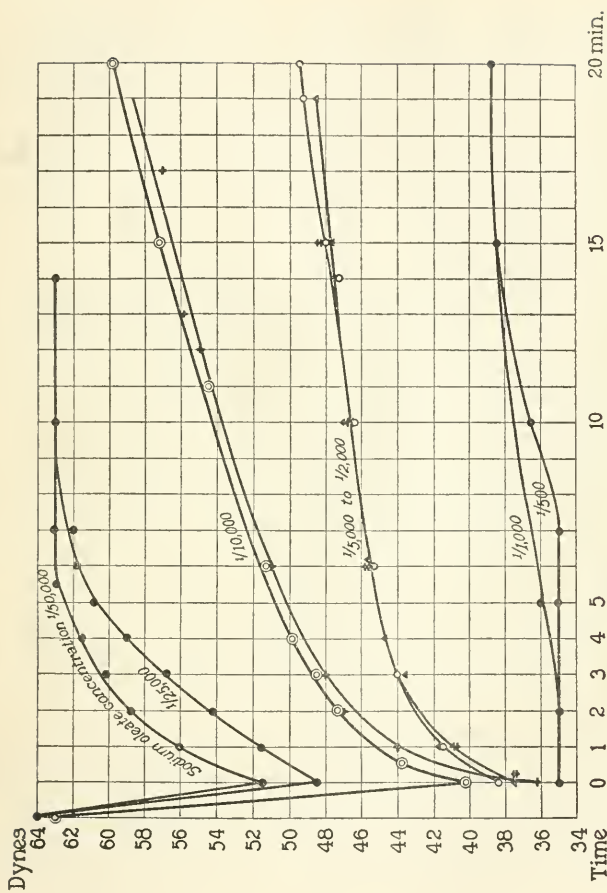
Temperature 22°C.

In every case, different amounts of sodium oleate were added to 1.9 gm. of serum solution. Each drop of sodium oleate weighed 0.019 gm.; 1, 2, 3, or more drops were added. Isotonic saline solution was used for the dilutions.

Time.	Surface tension.							
	Concentration of sodium oleate.							
	1/50,000	1/25,000	1/10,000	1/5,000	1/2,500	1/1,000	1/1,000	1/500
	dynes	dynes	dynes	dynes	dynes	dynes	dynes	dynes
Before addition of sodium oleate.....	63.0	63.0	62.5	62.6	62.5	61.2	63.0	63.0
After " " " "	51.5	48.5	40.2	38.4	36.5	36.5	34.3	34.2
" 1 min.....	56.0	51.6	46.5	41.5	40.9	41.5	34.3	34.5
" 2 "	58.5	54.2	47.3	43.0	42.9	42.9	34.5	34.5
" 3 "	60.2	57.0	48.5	44.0	44.0	43.7	34.5	34.5
" 4 "	61.5	59.0	49.8	44.5		44.6	34.0	
" 5 "	63.0	60.8	50.5	44.7	45.0	45.0	36.0	
" 6 "	63.2	61.8	51.3		45.4	45.4		
" 7 "						45.8		
" 8 "		62.2						
" 10 "	61.0	63.0		46.4	46.6	46.6		
" 11 "			54.5					
" 13 "								38.6
" 15 "		61.0		48.0	48.0	47.5	38.4	38.6
" 16 "			57.9					
" 21 "			57.9					
" 30 "			61.0	51.6				
" 1 hr., 30 min.....			58.0	52.3			39.5	39.2

protein and other colloidal substances of the serum, and of their physical behavior when placed in contact with other free molecules.

In order to study the process of recovery of surface tension of serum solutions quantitatively, varying amounts of sodium oleate in solution were added to the same quantity of serum solution at 1/10 dilution. The results are given in Table VII and Text-fig. 8.



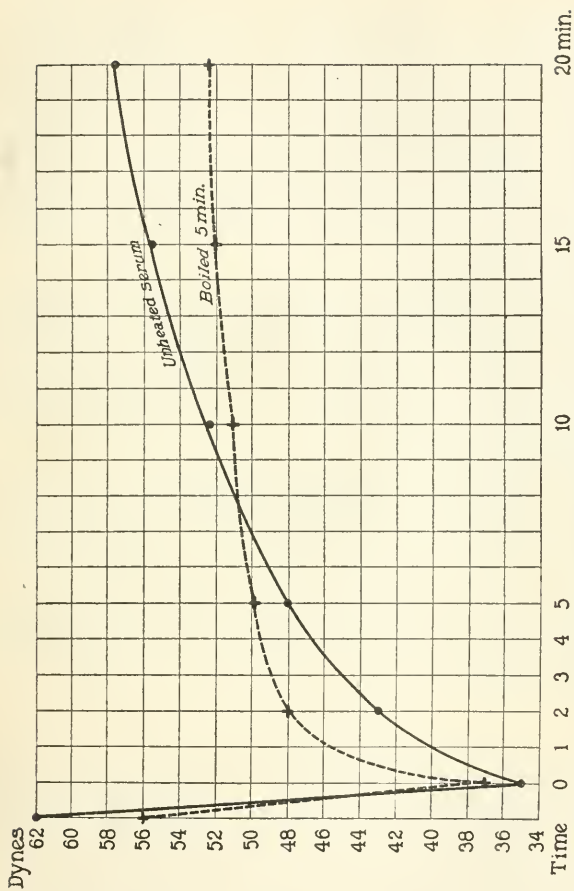
TEXT-FIG. 8. Rise of surface tension of a 1/10 serum solution after the addition of different amounts of sodium oleate.

It is decidedly important whether the solution is stirred or not after the addition of sodium oleate, or whether the surface-active substance is added powdered or in a concentrated or dilute solution. In other words, the results will be different if the concentration of 1/1,000 of sodium oleate is obtained by adding 1 drop of a 1/10, or 10 drops of a 1/100 solution of sodium oleate to the serum, compensation being made for the different amount of liquid. Generally speaking, the antagonistic action of the serum is more powerful when the sodium oleate is less dilute. It is strongest for pure powdered sodium oleate.

3. *Action of Heat.*—Heat modifies the properties of serum profoundly, even at a relatively low temperature such as 56°C. However, although the changes are important as far as the biological properties are concerned, they are undetectable to the chemist and the physicist. Heated serum is no longer the same, but were it not for biological tests, such as the study of hemolytic power, it would be impossible to differentiate it from unheated serum. Experiments on the recovery of the surface tension of serum solutions show that generally when a serum has been heated, the beginning of the recovery is more rapid, but that after 15 or 20 minutes, sometimes less, its curve crosses that of the recovery of the unheated sample, and remains below it. The serum acts practically in the same way whether heated before or after dilution. When heated before dilution, the phenomenon is slightly more marked. It was already known that dilution had a protective action on serum. The higher the temperature, the more marked the phenomenon, whether the serum was diluted in pure water or in saline solution. An idea of this phenomenon is given in Text-fig. 9.

In order to compare the sensitivities of the physiological and of the physical method, two samples of a 1/10 serum solution in saline solution were studied from the standpoint of the lowering of surface tension after the addition of sodium oleate. One sample was boiled in a water bath for 5 minutes, the other was not. Clark, Zinck, and Evans³ observed that, after boiling, the protective action of human serum against hemolysis of red cells (guinea pig) by sodium oleate

³ Clark, H. M., Zinck, R. H., and Evans, F. A., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 328.



TEXT-FIG. 9. Rise of surface tension of a 1/10 serum solution before and after heating 5 minutes at 100°C.

was decreased. In other words, it required a smaller concentration of sodium oleate to produce the same degree of hemolysis (1/50,000 with fresh serum, 1/65,000 with boiled serum). We were unable to check up these results by using rabbit serum and hen cells, but our measurements showed that the surface tension of the boiled serum solution was inferior by 10 dynes to that of fresh serum, which would suffice in itself to explain a difference, and that the recovery, although more rapid in the first 8 minutes, was much below that of the fresh serum at the end (Text-fig. 9.) When using serum solution in distilled water (dilution 1/20), the action was similar (Text-fig. 10).

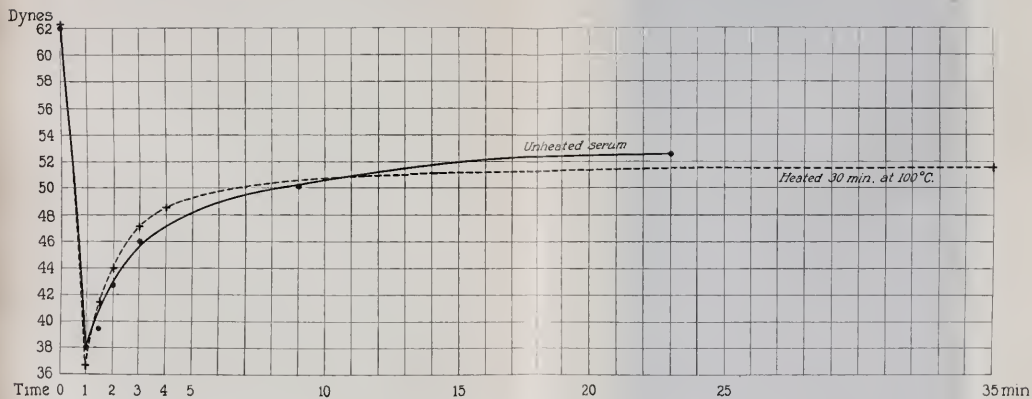
In this case, the study of the surface tension shows that the phenomenon is dependent on two factors; the initial value of the surface tension plays one part, and the antagonistic action of the serum another. The advantage of the physical method was that it decomposed a complex phenomenon into its elements, introduced the factor time, and did not depend on such an unreliable standard as hemolysis of red cells.

The considerable modifications brought about in the serum by temperature are also very strikingly illustrated by Figs. 1 to 4. These are photographs of the crystallization of four samples of a 1/10 solution of serum in saline solution, to which 1/10,000 of sodium oleate had been added. The first sample (Fig. 1) was unheated. The second (Fig. 2) was heated at 56°C. for 2 hours. The third (Fig. 3) was heated at 70°C. for 1 hour, and the fourth (Fig. 4) was boiled for 5 minutes. These phenomena will be studied in another paper.

III.

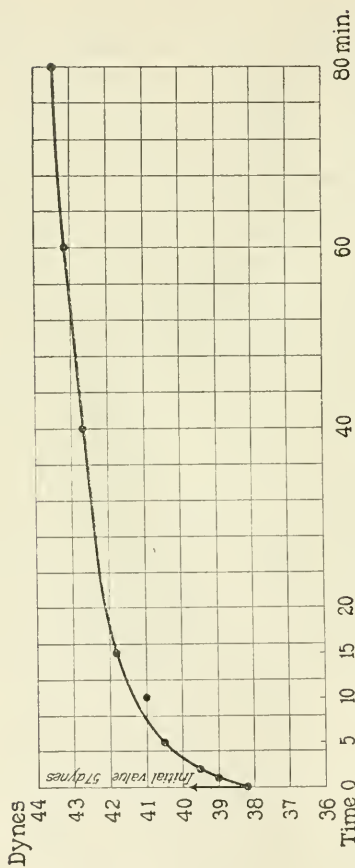
DISCUSSION.

All these facts may be accounted for by simply assuming that this phenomenon of recovery is not due to a special substance but to the adsorption of the surface-active molecules by the large colloidal micellæ of the serum. Comparison of the curves of recovery with ordinary curves of adsorption makes this plain, but an attempt was made to obtain the same phenomenon with inert colloidal solutions, such as gelatin and gum arabic. If our hypothesis

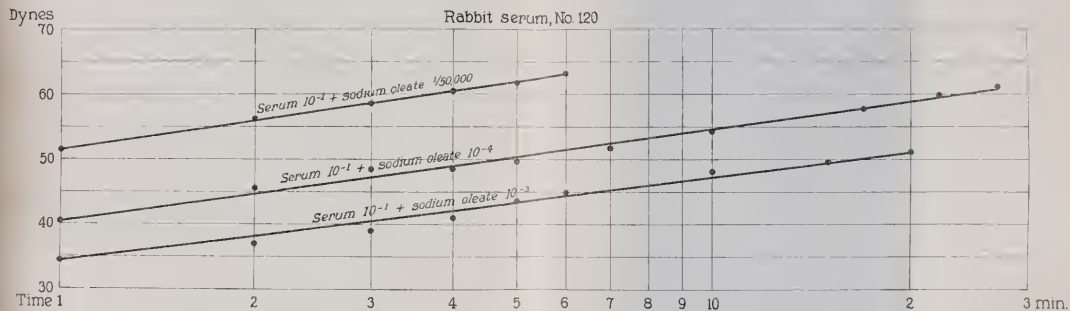


TEXT-FIG. 10. Rise of surface tension of a 1/20 serum solution in distilled water before and after heating 30 minutes at 100°C.

were correct, the same recovery should take place as soon as *any* colloidal particles should be present in an aqueous solution whose surface tension had been lowered by the addition of sodium oleate. The order of magnitude of the phenomenon depending mainly on the size of the particles, a much slower process was expected. Experiments showed the correctness of the views expressed above (Text-fig. 11), solutions of gelatin, egg albumin, and gum arabic acting in a way absolutely comparable to that of serum. When sodium oleate, powdered or in highly concentrated solution, is added to the serum, without much stirring, the oleate spreads all over the free surface, forming large aggregates of molecules which are rapidly adsorbed by the serum micellæ of the surface layer, some of them becoming heavy enough to fall to the bottom. At a certain optimum concentration, almost all the oleate molecules are being adsorbed; that is, withdrawn from the surface. They form a thick coating around the molecules or group molecules of serum, which tends to force them apart a little more than they were before. This explains why the surface tension, after recovery, often reaches a slightly higher value than before the addition of oleate. However, a great number of micellæ in the bulk are free from sodium oleate molecules and they become progressively adsorbed in the surface layer, thus lowering slightly the surface tension, while the sodium oleate-coated micellæ remain in the bulk or are precipitated, and the surface tension falls back to normal. On the contrary, should a dilute solution be used, the oleate molecules are very mobile. No large aggregates are formed, the solution mixes readily with the serum, the surface-active molecules are brought near every serum molecule in the bulk, adsorption follows, but the result is that *all* serum micellæ carry a certain amount of oleate molecules so that the recovery is slow and the surface tension may never reach as high a value as in the case in which concentrated oleate solution was used. Of course, in the case of powdered or highly concentrated oleate, stirring will produce the same effect as if the solution were more diluted. But when the solution is stirred only slightly or not at all, it is easy to observe the occurrence of a visible and rather coarse precipitate, while if the solution has been vigorously stirred, it remains clear and merely opalescent. When a solution of 1/10 of



TEXT-FIG. 11. Rise of surface tension of a 1/50 solution of gum arabic in saline solution 0.9 per cent after the addition of 1/30,000 of sodium oleate.



TEXT-FIG. 12. Rise of surface tension of a 1/10 serum solution after the addition of different amounts of sodium oleate (curves of Text-fig. 8), plotted on semilogarithmic paper.

oleate is used, films are seen floating at the surface. This never occurs with a solution of 1/100.

In our experiments, the phenomenon of recovery was not a simple function of the concentration of sodium oleate; whether the concentration of oleate was 1/5,000 or 1/2,000, the rate of recovery was almost identical at the beginning, while there was a considerable difference between the rates of recovery of solutions at 1/10,000 and 1/5,000 (Text-fig. 8). The same gap exists between 1/2,000 and 1/1,000 while 1/1,000 and 1/500 act practically in the same way. Too little is known about the structure of the serum molecules and the intimate mechanism of the surface tension to attempt a quantitative explanation of these facts. There is no doubt but that the solution of the problem will be found in the knowledge of the interactions of the group molecules in the surface layer. The curves (Text-fig. 8) correspond to possible geometrical arrangements of the group molecules, resulting in certain optima stray fields. These facts will only be cleared up some day by a thorough study of the physics of the molecules, probably on the basis of Langmuir's theory.⁴

Some of the curves of recovery of surface tension were plotted on semilogarithmic paper and appear as straight lines (Text-fig. 12). This indicates that the process follows the compound interest law, and may be expressed by an equation of the form

$$\gamma = ae^{bx}$$

similar to that established for the phenomenon of spontaneous lowering of surface tension,^{1,2} and adsorption in general.

IV.

CONCLUSIONS.

1. The equilibrium of the serum corresponding to its normal minimal surface tension is as stable and difficult to break, under ordinary conditions, as the osmotic tension equilibrium. The addition of a strong surface-active substance (sodium oleate, glycocholate, or taurocholate), will not lower it definitely, unless the substance is present in large amounts and in solution. After the first rapid drop

⁴ Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1848.

has occurred, a process of recovery takes place, which brings back the normal surface tension in a short time (from 2 to 6 minutes in the case of pure serum). As a drop in the surface tension of the serum of animals may be very injurious to the red cells, this process of recovery is a normal one of defense in all cases in which surface-active substances (bile) are set free in the blood.

2. When diluted, the serum shows the same phenomenon to a smaller extent; the time of recovery is very much longer and the final surface tension is always lower than the original value. At a dilution of 1/10,000, no recovery takes place, the dilution being too high to overcome the lowering action of 1/10,000 of sodium oleate.

3. The recovery is stronger when the surface-active substance is added powdered or in a highly concentrated solution, and not stirred.

4. The recovery does not seem to be inversely proportional to the concentration of sodium oleate, when added superficially. Doubling the concentration at 1/2,000, for example, gives the same curve of recovery. This happens under certain conditions; namely, when the liquid is not stirred after the addition of sodium oleate.

5. This recovery is due to a purely physical phenomenon, namely adsorption, and is not specific for the serum. Other colloidal solutions, such as gum arabic, egg albumin, gelatin, and silver and gold sols, show it, only to a smaller degree. The process of recovery follows a logarithmic law in all cases, expressed by an equation of the form

$$\gamma = ae^{bx}$$

6. Temperature affects this phenomenon. At first it enhances it, but finally decreases it. This would seem to connect the loss of the property of the serum known as complement in a serum with a modification of the physical properties of this serum. This phenomenon is being investigated further.

EXPLANATION OF PLATE 2.

FIGS. 1 to 4. Action of temperature on the crystallization of serum solutions, concentration 1/10 in saline solution 0.9 per cent (rabbit serum).

FIG. 1. Unheated.

FIG. 2. Heated at 56°C. for 2 hours.

FIG. 3. Heated at 70°C. for 1 hour.

FIG. 4. Heated at 100°C. for 5 minutes.

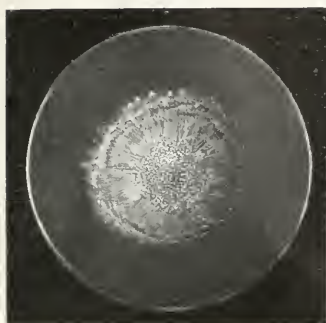


FIG. 1.

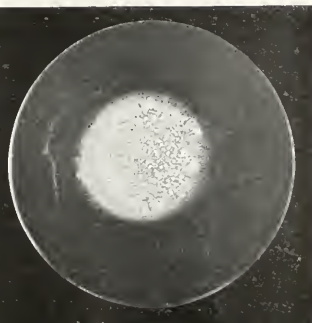


FIG. 2.

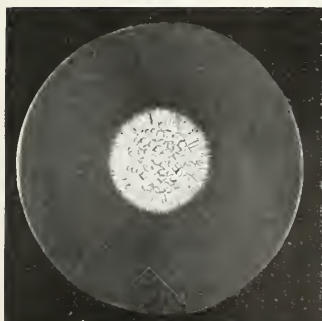


FIG. 3.



FIG. 4.

(du Noüy: Surface tension of serum. 111.)

INFECTIOUS MOTOR PARALYSIS IN YOUNG RABBITS.*

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PLATES 3 AND 4.

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The paralysis described in this article was observed during experiments planned to confirm, if possible, the rat and flea transmission theory of infantile paralysis as outlined by Richardson.^{1,2} The investigations began about February 1, 1919, and continued over a 10 month period. In general it was planned to infect young rabbits through intracerebral inoculations with the virus of infantile paralysis and then to determine whether the infection could be transferred from the sick animal to the healthy through the bites of fleas.

For the primary intracerebral inoculations four viruses were secured.³ The rat flea (*Ceratophyllus fasciatus* Bosc⁴) was used in most of the experiments. In a few cases dog and cat fleas (*Ctenocephalus*

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¹ Richardson, M. W., The rat and infantile paralysis—a theory, *Boston Med. and Surg. J.*, 1916, clxxv, 397.

² Richardson, M. W., The rat and infantile paralysis—a theory, *Am. J. Pub. Health*, 1918, viii, 564.

³ Two straight monkey viruses were kindly furnished by Dr. George W. McCoy of the United States Hygienic Laboratory, Washington, D. C., and two mixed monkey viruses by Dr. Simon Flexner of The Rockefeller Institute for Medical Research.

⁴ Craighead, E. M., Observations on certain Siphonaptera, *Entomol. News*, 1921, xxxii, 303.

canis Curtis, and *Ctenocephalus felis* Bouche) were used. In the preliminary intracerebral inoculations from 1 to 0.5 cc. of a suspension of glycerolated spinal cord in $\frac{8}{1}$ salt solution was used.

Fifteen rabbits were inoculated intracerebrally with virus from the Hygienic Laboratory without pathological effect. Eight guinea pigs and three rabbits were inoculated intracerebrally with Rockefeller Institute virus, and all remained well but one rabbit, No. 12. Beginning 12 hours after inoculation fleas were allowed to feed alternately first on inoculated and then on normal animals, and this sequence was continued daily until the fleas died, or until the secondary animal showed paralysis. About 150 fleas were used at each feeding.

March 20, 1919. Rabbit 12 (5 weeks old) was inoculated intracerebrally with 0.1 cc. of a suspension in $\frac{8}{1}$ salt solution of glycerolated spinal cord. March 25. The animal showed some tremor and developed slight paralysis of the fore quarters. April 22. Died. The pathological findings were negative.

Fleas which had bitten Rabbit 12 were transferred daily to Rabbits 12 B and 18 (7 days old), and the latter developed typical paralysis dying 33 and 40 days respectively after their first flea bites. There was never any possible physical contact with Rabbit 12. Under similar conditions nine other rabbits became paralytic and died, the intervals between the first flea bites and death ranging from 9 to 40 days.

It seemed, therefore, highly probable that a paralytic disease having its origin in human infantile paralysis had been induced in young rabbits and transferred from animal to animal by the bites of fleas. The fallacy of this conclusion was quickly demonstrated, however, by the proper control experiment, in which three normal rabbits were simply placed in cages with the sick rabbits, the intermediate biting of fleas being omitted. Two of these control rabbits developed paralysis and one died.

Thus we had to do, presumably, with a paralytic disease in young rabbits spread by simple contact. Further investigation, moreover, showed it to be associated with a large organism which, as will be seen later, can only by the remotest possibility, have anything to do with human infantile paralysis.

Clinically this rabbit disease is characterized by a persistent drowsiness, followed in most cases by tremor shortly before the development of paralysis. The paralysis may be slight or marked, general or localized, and the mortality is high.

During the disease period stained blood smears were examined daily, but nothing suggestive of microorganisms was found. In ten cases cultures were made upon every available medium from brain, cord, kidney, spleen, and liver but no growth occurred. Cultures and smears from the digestive systems and salivary glands of fleas gave negative results. Attempts to transmit the disease through the bites of bedbugs and mites were negative. Intracerebral inoculations of sedimented urine, also of cord emulsions from sick rabbits, reproduced the paralytic disease in other rabbits.

Examination of the infected rabbits showed the following gross and microscopic factors and conditions.

Spinal Cord and Brain.—The cords from nine animals, together with the brains of five of them, were examined. No definite gross lesions were apparent except softening of the cord.

Microscopic sections show small focal lesions in both the gray and white matter, which rarely occupy an area as large as the field of an oil immersion lens (Fig. 1). They consist in infiltration by small inflammatory cells with rounded or irregular shaped nuclei. Polymorphonuclear leucocytes do not take part in the infiltration. The nerve cells in the infiltration areas are diminished in number or absent. Occasionally a degenerated or necrotic nerve cell is present. The foci of infiltration in the white matter are associated with capillary blood vessels.

The lesions are present in one or more sections of the cord, or cord and brain, of each of the nine animals. In a cross-section of a cord one or more lesions (up to about half a dozen) may be present. In a few instances there is mononuclear cell infiltration about the vessels of the pia mater.

By appropriate staining methods peculiar bodies are demonstrable in most of the lesions. These are considered to be microorganisms and will be referred to as such. They are elongated, sharply and smoothly outlined, with rounded or conical ends, and are slightly variable in shape and dimensions. Their length and thickness are

probably never more than 4 and 1.5 microns respectively. As a rule, they do not stain homogeneously, but show one or two lighter staining areas. In general appearance they closely resemble bacilli. They stain by Gram's method and with methylene blue. Wright's stain does not show any nuclear structure in them and stains them only faintly. They are acid-fast to a certain extent. The best method of demonstrating them consists in first staining the section with carbolfuchsin (diluted 1:4), mordanting and decolorizing with undiluted formaldehyde,⁵ and counterstaining with methylene blue. By this method the microorganisms are stained red and the nuclei of the cells blue.

The microorganisms are found in varying numbers in the lesions. They occur scattered among the infiltrating cells and occasionally, in large number, in nerve cells, or in compact aggregations in smoothly outlined rounded or oval spaces in the tissue, of about the size of a nerve cell. The cytoplasm of a nerve cell containing the microorganisms may be vacuolated and the cell body more or less transformed into a shell (Fig. 2). The spaces filled with microorganisms seem to represent destroyed nerve cells. In one instance a nerve cell in a spinal ganglion is thus apparently represented by a compact mass of the microorganisms.

Nerve cells invaded by the microorganisms and the aggregations in spaces are not always closely associated with the focal infiltration by inflammatory cells, and there may be no evidence of inflammatory reaction about them. The appearances seem to indicate that invasion and destruction of nerve cells is the first step in the development of some of the focal lesions.

Kidney.—The kidneys from five rabbits all showed essentially the same lesions, which were either not apparent to the naked eye or appeared as grayish spots and streaks on the cut surface. The lesions are chiefly in the medulla. They consist essentially of multiple small foci of infiltration by small cells with round, indented, or fragmented nuclei, and are marked by degeneration and disappearance of the epithelial cells in the infiltrated areas. Microorganisms like

⁵ The use of formaldehyde for this differential staining of the microorganisms was suggested by Dr. William H. Smith, who has long employed it successfully as a mordant in staining certain bacteria.

those in the central nervous system are present. They occur chiefly in closely packed aggregation in epithelial cells and in the lumina of tubules in varying numbers along with free cells and detritus (Fig. 3). In the denser infiltrated areas they are generally few or not demonstrable. The number of epithelial cells harboring the microorganism is relatively small and they are scattered. In sections stained with fuchsin and methylene blue, as above described, there may be seen in tubules, along with the characteristic red-staining microorganisms, blue-staining bodies of varying shape and size which seem to be degenerated forms.

The microorganisms were found in the urine during life. The staining method above described served to differentiate them from the bacilli present, which stained blue (Fig. 4).

Spleen.—Sections of the spleen from four rabbits show the presence in the pulp of large mononuclear phagocytic cells containing what seem to be degenerate forms of the microorganisms. These are variable in size and shape and show an affinity for methylene blue instead of fuchsin.

Liver.—A section from only one animal was examined and this showed two or three small focal lesions.

Myocardium.—Sections from two animals were examined and a few focal lesions found.

SUMMARY.

1. The attempt to infect young rabbits and guinea pigs with material containing in all probability the virus of human infantile paralysis failed.

2. Failure to infect the primary animals almost of necessity brought failure with the secondary flea-bitten animals. It is, however barely conceivable that a non-infectious form of an organism might circulate in the blood of the primary animal and that this form, through development in an intermediate host, the flea, might become virulent for the secondary flea-bitten animal.

3. Incidentally, and presumably accidentally, a paralytic disease was observed in young rabbits associated with the presence of an organism showing certain definite characters. So far as we know this paralysis and the associated organism have not been previously described.

4. This organism is found widely distributed in the organs of the affected animals and can be demonstrated in the urine. The active destruction by the organism of the nerve cells of the spinal cord is particularly striking, and gives complete explanation for the paralysis observed clinically.

5. With the organism present in the urine the spread of the disease by contact can be easily understood.

6. The transfer of the infection from animal to animal by fleabites is possible but not probable.

7. The nature of the observed organisms is in doubt. They represent probably an intermediate stage in the life history of some protozoan parasite.

EXPLANATION OF PLATES.

PLATE 3.

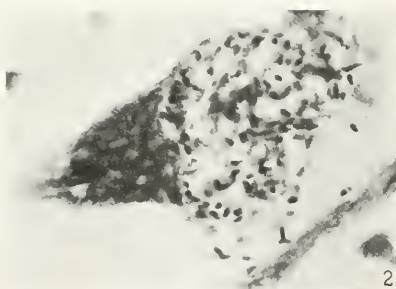
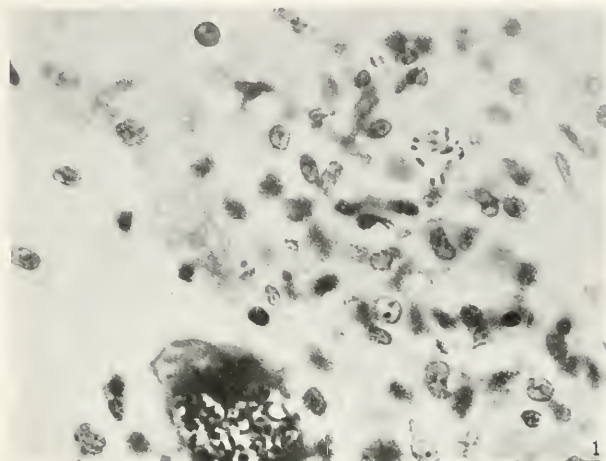
FIG. 1. A focal lesion in the spinal cord showing the infiltration by cells, and the microorganisms, many of which are in a degenerated nerve cell at the lower margin. $\times 1,000$.

FIG. 2. Nerve cell of the spinal cord, degenerated and invaded by many of the microorganisms. A few microorganisms lie outside of the cell. $\times 1,000$.

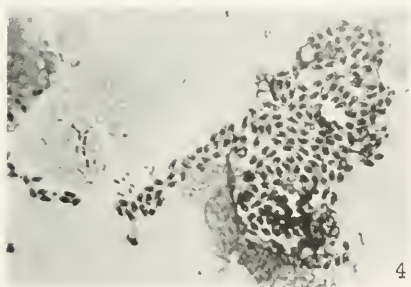
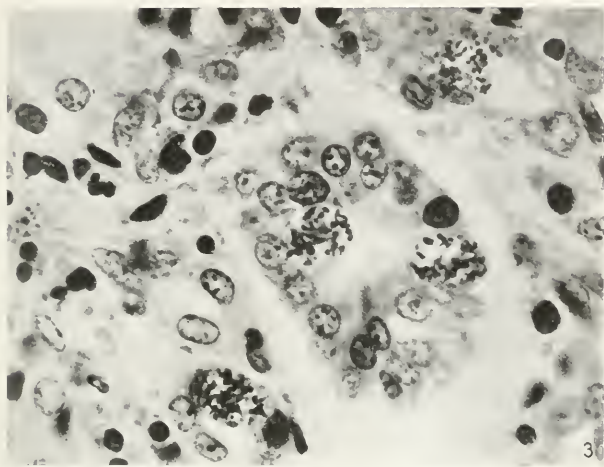
PLATE 4.

FIG. 3. Groups of microorganisms in four epithelial cells of the kidney. A sparse inflammatory cell infiltration is also shown. $\times 1,000$.

FIG. 4. A mass of microorganisms in a smear preparation from a urinary sediment. Some bacilli are also present. $\times 1,000$.



(Wright and Craighead: Infectious motor paralysis.)



(Wright and Craighead: Infectious motor paralysis.)

THE STRUCTURE AND DIFFERENTIATION OF THE SPECIFIC CELLULAR ELEMENTS OF THE PARS INTERMEDIA OF THE HYPOPHYSIS OF THE DOMESTIC PIG.*

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PLATE 5.

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Since the discovery by Oliver and Schäfer that the hypophysis contains a specific substance which causes, when injected into the blood serum, a rise of blood pressure, much interest has been displayed in the question as to the source of this material and the mode of its discharge from the gland.

The observations of Howell, which have been amply confirmed, definitely established the origin of the pressor material exclusively from the posterior lobe of the gland, but left unsettled the question whether the active portion of this lobe is the epithelial investment derived from the pouch of Rathke, or the nervous portion derived from the brain.

Many efforts have been made to recognize histologically the antecedents of the secretion in the several parts of the posterior lobe. Of particular interest in this connection are the observations of Herring (1908), since they have given rise to a conception of posterior lobe secretion which has been supported by later experimenters, and widely adopted by those interested in the problems of internal secretion.

Herring studied in particular the relations of the pars intermedia, or epithelial investment, to the nervous substance of the posterior lobe. In the former he saw frequently vesicles wholly or partly formed of epithelial cells and filled with a material which resembled, under the microscope, the colloid of the thyroid vesicles. In the pars nervosa also numerous small masses of a hyaline material sometimes homogeneous, sometimes finely granular, were found. These hyaline masses were found throughout the substance of the pars nervosa and gave the impression of materials flowing towards the recess of the infundibulum, in the cavity of which also similar deposits were found. Herring expressed himself with considerable

*The expenses of this investigation were provided by a fund donated by Mrs. Nettie F. McCormick of Chicago, to whom the authors wish to express their grateful appreciation.

reserve concerning the nature and origin of these hyaline bodies but considered two possibilities. He noted that they were found frequently between the ependymal cells near the ventricular cavity. He considered this as suggestive of their being the product of ependymal cells, but remarked that the hyaline was not always confined to this situation since it was frequently found among the cells of the epithelial investment, a fact pointing strongly to its origin from the epithelial cells. In some cases the hyaline bodies were apparently surrounded by a layer of cells resembling endothelium, as if they were contained in lymphatic vessels.

In a later study of the changes in the hypophysis produced by complete thyroidectomy in rabbits and dogs, Herring found proliferation of the cells of the pars intermedia with invasion of the pars nervosa, and an enormous quantity of granular, hyaline, or colloid bodies in the body of the pars nervosa and in the laminae forming the floor of the third ventricle of the brain. Concerning these changes he says: "The significance of these changes is as yet undetermined. The colloid bodies appear to arise from the epithelial cells of the pars intermedia, and their extensive production to be an exaggeration of a normal process."

Crowe, Cushing, and Homans (1910) in their study of the results of partial and complete hypophysectomies in the dog reached similar conclusions. Referring to Herring's observations they say: "and we agree with him that the histological appearances point strongly toward the fact that this is an actual secretion and one which appears to be the product of activity of the cells of the epithelial investment (pars intermedia). The material seems to pass toward the infundibulum and in many cases may actually be seen passing into the cavity of the third ventricle." According to these authors neither pure intermedia substance nor the colloid of the pars intermedia vesicles has any pressor effect. They suggest, therefore, that the pars nervosa activates the hyaline bodies "at least if they are in any way related to the glandular colloid" during their passage toward the infundibular cavity.

Cushing and Goetsch (1910-11) meanwhile have confirmed the results of Herring's observations as to the increase of hyaline bodies, etc., after thyroidectomy, and found a similar increase after a number of other experimental procedures; for example, after total pancreatectomy, mechanical injuries, partial hypophysectomies, and after experimental obstruction of the stalk by means of a silver clip applied about it. These authors accept unreservedly Herring's conception of a secretory product passing through the pars nervosa and believe that it is discharged into the cerebrospinal fluid. They also state definitely that they believe the colloid accumulation in newly formed epithelial vesicles is merely a precursor of the hyaline bodies, though the latter apparently may originate by a direct transformation of individual wandering epithelial cells. These authors also obtained with human cerebrospinal fluid concentrated by evaporation, injected intravenously in dogs and rabbits, unmistakable evidences of the presence in the fluid of a pressor substance similar to that obtained from extracts of the posterior lobe of the hypophysis.

Carlson and Martin (1911-12), on the other hand, failed to get any pressor response whatever from the intravenous injection of dog cerebrospinal fluid into dogs. It must be noted, however, that these workers did not concentrate the cerebrospinal fluid prior to testing it, as did Cushing and Goetsch. Carlson and Martin suggest that the pressor response obtained by Cushing and Goetsch may have been due to their using foreign, pathological, and concentrated cerebrospinal fluid.

The net result of these various researches is an expansion of Herring's views. The pars intermedia elaborates a specific secretion which it discharges into the pars nervosa either in the form of colloid or by the degeneration of pars intermedia cells which have migrated into the pars nervosa. The secretion appears in the pars nervosa as hyaline bodies either free in the interstices of the glia or inside of lymphatic channels. It finally reaches the ventricle and is discharged into the cerebrospinal fluid. This product contains the pressor substance of Oliver and Schäfer. It is to be noted, however, that Herring recognizes the alternative possibility that the hyaline bodies may be a product of the activity of ependymal cells.

In order to establish this hypothesis it is necessary to prove that the active substance of the secretion is present in the epithelial structure from which it is supposed to take origin and also in the nervous tissue through which it passes en route to the ventricular cavity as well as in the cerebrospinal fluid itself. Unfortunately it is not easy to separate the colloid product of the pars intermedia from the epithelial cells which surround it or the pars nervosa from the pars intermedia. It is not surprising, therefore, that the results of these inquiries have not yielded wholly concordant results.

Crowe, Cushing, and Homans, as already mentioned, found the colloid obtained from cysts of the pars intermedia, and pure pars intermedia substance scraped off the pars nervosa, equally inactive, and adapted these findings to their hypothesis by assuming that the secretion was activated during its passage through the posterior lobe.

Lewis, Miller, and Matthews (1911) found the posterior part of the anterior lobe, pars intermedia, and pars nervosa, all active as regards the pressor substance, but found the colloid contained in the residual lumen of the gland only active when there was desquamation or autolysis of pars intermedia cells in the colloid. It must be pointed out, however, that the material contained in large cysts is hardly comparable to the colloid observed in small vesicles of the pars intermedia, since the former are lined in large measure by epithelial cells which have vestigial stomodeal characters, and it may be reasonably doubted whether any of the pars intermedia secretion of a specific nature is discharged into these cavities.

Biedl reports that in a number of cases he was able to separate an inactive pars nervosa from the surrounding pars intermedia, though he declares that this can rarely be accomplished on account of the anatomical difficulties involved. Biedl also found pars intermedia substance active in producing a pressor response, thus confirming the claim of Lewis, Miller, and Matthews.

On the basis of these investigations we may now consider that the presence of the pressor substance in the pars intermedia is established but that its relation to the colloid material of the pars intermedia or to the hyaline and granular masses of the pars nervosa remains a subject for further investigation.

The theory of the relation of colloid derived from pars intermedia to the hyaline and granular masses of the pars nervosa rests on the very unconvincing evidence afforded by the similarity in appearance in microscopic preparations, the frequent proximity of hyaline masses to epithelium, the fact that colloid in vesicles increases under experimental conditions which also increase the content of hyaline, and the fact that the vesicles containing colloid have not always a complete epithelial wall.

The theory that hyaline is derived from degenerating epithelial cells which have invaded the pars intermedia does not adequately explain the presence of hyaline in sites where epithelial invasion rarely occurs, as, for example, in the postoptic lamina and in the portions of the pars nervosa remote from the stalk. Furthermore, hyaline is abundant in the hypophysis of animals in which the pars intermedia is sharply delimited from the pars nervosa, and in which there is seldom, if ever, any epithelial invasion.

It is apparent from the foregoing review that Herring's theory of the mode of posterior lobe secretion as expanded by Cushing and his coworkers rests on a very precarious foundation. Its wide acceptance by investigators is doubtless due to the lack of evidence supporting the equally plausible hypothesis that the pars intermedia, like other endocrine organs, secretes its hormones directly into the circulating fluids. It is well to remember, however, that in constructing the Herring hypothesis as modified by subsequent observers certain assumptions are made which are themselves open to doubt. These are as follows: (1) Only epithelial cells are capable of secretory activity. (2) Amorphous masses occupying contiguous situations in histological preparations are chemically similar and are derived from a common source. (3) Masses forming a row in a microscopic preparation do so because they are moving in the same direction towards a common goal. (4) The increase in the amount of a given product under experimental conditions is an exaggeration of a normal process, a dis-

turbance of the equilibrium between secretion and export. In Cushing and Goetsch's silver clip experiment there is, in addition to obstruction of the recessus infundibuli, actual trauma and interference with the blood supply. The really cogent facts in support of the hypothesis are the presence of a specific pressor substance in the cells of the pars intermedia as demonstrated by Lewis, Miller, and Matthews, and confirmed by Biedl, and the presence of pressor substance in the cerebrospinal fluid. The latter is, however, contested by Carlson and Martin, and Wassing, working under Biedl's direction, was unable to demonstrate clearly the presence of vasoconstricting substances in the cerebrospinal fluid by the frog perfusion method.

The present paper is the result of a research undertaken to test the Herring-Cushing hypothesis, as compared with the alternative hypothesis of direct activity of the pars intermedia occupied with direct export of its product into the vascular channels. Before proceeding to a description of the technique employed and of the results obtained, in order that the point of view of the work may be understood, it may be well to point out that glandular cells of different types can only be distinguished from one another with certainty by the observation and study of the actual products of their activity. In the case of a mixed gland such a study must, of necessity, be made by microscopic methods applied to the living gland when possible and supplemented by observations on the precipitability of these products and on their staining reactions, or microchemical reactions if such be available in the fixed condition. The precarious foundation on which identification of gland cells by purely morphologic criteria rests is sufficiently illustrated by the long discussion not yet terminated of the nature of the demilunes of the salivary glands, and the error, most persistent in our text-books of histology and physiology, of describing the pyloric glands as serous glands. Accordingly the first question which presented itself was as follows: Can the pars intermedia cell be distinguished as a physiological unit by the nature of its chemical products from each and every type of cell in the anterior lobe of the hypophysis? This being answered in the affirmative and the characters of this product determined, the next phase of the problem is to determine whether there is any correlation in the embryo between the time of appearance of pressor activity in the hypophysis and that of

the specific product of the pars intermedia cells. Of necessity, the work must include a study of the other parts of the hypophysis as well as of the pars intermedia.

Technique.

Successful handling of hypophysis from the technical standpoint requires that the organ should be removed with the utmost speed as soon as the animal is dead and transferred with as little mechanical injury as possible to a suitable fixing fluid. We have found that postmortem changes proceed with great rapidity, particularly changes which involve the secretory antecedents in the cells of the pars intermedia. Also mechanical injury by pinching with forceps, compression, or traction cause obvious changes in cytological structure. In handling the adult material a certain amount of delay was inevitable on account of the abattoir processes, but usually it was possible to have the hypophysis in the fixing fluid within 5 minutes from the time the hog was hoisted on the wheel. As soon as the animal was bled, the top of the skull and brain were removed and the hypophysis was carefully dissected out of the sella turcica and dropped into a large quantity of fixing fluid. After a lapse of a few minutes, sufficient to stiffen the surface of the organ, the gland was removed with a spoon and carefully divided in the sagittal plane. The halves were allowed to remain in the fixing fluid for 24 hours in the dark, then dehydrated, cleared in bergamot, and imbedded in paraffin according to the usual practice.

For fixing fluids, formaldehyde 40 per cent, aqueous chrome sublimate (Zenker's fluid without acetic acid), and formalin-Zenker (Zenker's fluid without acetic acid nine parts, neutral formalin one part) were employed.

The following staining methods gave the best results.

1. The Acid Fuchsin-Acid Violet Method.—The stain consists of a mixture of 70 cc. of 1.7 per cent solution of Grüber's acid fuchsin in water, to which is added 30 cc. of a saturated aqueous solution of acid violet. After removal of the paraffin by benzene and transfer of the sections through alcohols to water, the sections are stained for 20 to 30 seconds in this mixture. They are then quickly blotted, dehydrated in anhydrous acetone, and cleared in anhydrous benzene. They are

next differentiated in a mixture of three parts of clove oil to one part of absolute alcohol, blotted, washed thoroughly in several changes of benzol, and mounted.

2. *The Neutral Safranine-Acid Violet Method.*—The dye is prepared as recommended by Bensley but is used in a different way. Sections 4 micra in thickness were cleared in benzene and passed through absolute alcohol to 70 per cent alcohol containing iodine, in which they remained until all mercury deposits were dissolved. The excess iodine was removed by further treatment with 95 per cent alcohol. Then 20 drops of the stock solution of the neutral dye were dropped on the alcohol-covered slide. 4 drops of distilled water were added and the whole was thoroughly mixed on the slide and allowed to remain for 40 to 50 minutes in the open room if the atmosphere was warm and moist or in a moist chamber if it was dry. The excess stain was poured off, the section blotted with a lintless filter paper, passed rapidly through 95 per cent alcohol to remove precipitated dye, dehydrated in absolute alcohol, and cleared in benzene. The sections were differentiated under the microscope with the oil of cloves-absolute alcohol mixture as described above. The beautiful differentiation of the cell elements of the anterior lobe after formalin-Zenker fixation obtained by this method is illustrated in Fig. 1.

3. *The Neutral Gentian Method as Described by Bensley.*—At this point it may be well to explain that the terms oxyphil, or eosinophil, and basophil as applied to the cells of the anterior lobe are not truly descriptive, since the cells in question stain with either acid or basic stains. It may even be questioned whether the cells described under these names in various animals or the same animal by different authors constitute a strictly comparable group. For this reason, in order to avoid confusion in our own identification of cell types, the following method was adopted. A section was first stained in safranine-acid violet. In this section a group of cells containing representatives of the different types of the anterior lobe was selected, carefully traced with the camera lucida, and each cell identified. The cover-glass was then removed, and the section restained with acid violet and acid fuchsin after extraction of the first stain. The cells were again identified and described, and the process repeated with neutral gentian and other stains.

To determine postmortem change, one half of the gland was placed in the fixing fluid and the other kept for 4 hours in blood serum at 18°C. Sections from the two halves were then compared after the same staining.

Cells of the Anterior Lobe.

In order to establish the fact that the pars intermedia is a functional unit in the hypophysis, it is necessary for comparison to describe and illustrate the cells of the anterior lobe. Five different types of cells are readily distinguished. Three of these are chromophil types and two chromophobe.

1. Chromophil cells.

(a) Type 1. Eosinophil, oxyphil, or acidophil of other investigators.

(b) Type 2. Basophil cells of other investigators.

(c) Type 3. A hitherto undefined cell forming the main mass of the darker band of tissue extending down from the stalk on the anterior surface of the gland.

2. Chromophobe cells.

(a) Chromophobe seen after formalin-Zenker and aqueous chrome sublimate fixation.

(b) Chromophobe seen after formaldehyde fixation.

There is little to be gained by an attempt at description of the cell types since they do not possess cytologic differences either cytoplasmic or nuclear which would suffice to distinguish them with certainty in the absence of specific staining reactions of their organized secretion products. The reader is, therefore, referred to Fig. 1, which shows a section of anterior lobe, formalin-Zenker fixation, stained in safranine-acid violet, showing chromophobes and Types 1 and 2 of chromophil cells. In general, the granules in Type 1 (oxyphil cells) are more diffusely distributed throughout the cell substance than in Type 2 (basophils), and in the latter when the secretory content of the cell is small, it is usually collected into two or more oval or comma-shaped masses which are quite characteristic of this cell in the pig, and serve to identify it whatever the staining method. In both the oxyphils and basophils, so called, alongside of the nucleus may be recognized a mass which never contains secretion granules though in well filled cells it may be obscured by them. This is the sphere. It is always surrounded by a veil of cytoplasm containing many fine mitochondria and in preparations fixed in formalin-Zenker often is detached from the rest of the cytoplasm in part by clear spaces which are apparently similar to the canal-like structure described by Holmgren and others at one pole of the nucleus in epithelial cells. This is the macula described by Addison (1917) in the rat and by Rasmussen (1921) in the woodchuck in the basophil cells. It is not exclusively the property of the basophil cells but may be more conspicuous in these cells in some species. Such a structure is also present in the cells of the pars intermedia. The mitochondria in the oxyphil and basophil cells of the pig tend to be aggregated about the sphere and to some extent about the periphery of the nucleus, though they may

be more dispersed in cells containing a small amount of accumulated secretion, and there are always scattered mitochondria throughout the mass of secretion. These scattered mitochondria mark the location of the residual protoplasm of the cell. The mitochondria are in reality coextensive with the protoplasm and are condensed where the protoplasm is continuous, dispersed where the protoplasm is dispersed by the accumulation in it of secretion product. This may be taken as a criterion of what is essentially cytoplasmic in these cells, as distinguished from the casual and variable products of cell activity. The mitochondrion never intrudes itself into actual spaces of the cytoplasm occupied by a secretion droplet or a group of them. The characters just described, while interesting, are of little use for cell identification, which must depend solely on the properties of the specific secretion products or granules. These differences will be discussed later.

Type 3 of the chromophil cells (Fig. 2) is found in a strap-like layer that extends down the front of the anterior lobe a variable distance from the stalk. It can be clearly recognized in the fresh gland by its darker color and greater transparency than the rest of the anterior lobe. In sections this mass is sharply delimited from the rest of the anterior lobe even though a few anterior lobe elements of the other types may intrude. This division of the anterior lobe contains more connective tissue than the rest, and it is composed of the cells of Type 3, intermingled among which are some other chromophil cells. These cells are especially conspicuous in sections of material fixed in formalin-Zenker and stained with either the neutral safranin compound or acid violet-acid fuchsin. In both cases they stain intensely blue. The cells tend to a polygonal outline in section rather than to the spherical. The nucleus, not markedly different in type from that of the other two types, is placed at one end of the cell, and the cytoplasm presents an irregularly vacuolated appearance. In addition, the cytoplasm is filled with blue-stained granules. These granules are not colored by the stains here used after fixation with formaldehyde, while those of Types 1 and 2 are fairly well preserved.

Among the chromophobe cells in general two types are seen, though it is not certain that they are morphologically distinct. The first includes all of those seen after formalin-Zenker and aqueous chrome

sublimate fixation and is characterized by the fact that the cytoplasm contains no secretion antecedent granules and retains less color than that of the chromophil cells (Fig. 1). The shape is irregular, being determined by external pressures. The second type seen in formalin-fixed preparations is large and tends to be spherical; and the cytoplasm takes a light diffuse stain, but a more intense stain than the smaller chromophobes, and it may contain a few large granules which stain with acid violet. The real nature and relationship of these cells remain undetermined and they are only mentioned here to establish the dissimilarity of the chromophobes and the cells of the pars intermedia.

Cells of the Pars Intermedia.

The cells of the pars intermedia (Fig. 3) (leaving out of consideration the more or less indifferent cells of the residual lumen) are of two sorts, one of which, the secretory cell of the pars intermedia in the strict sense, is different from every other cellular element in the hypophysis and by its presence serves to delimit the pars intermedia. The other type is the colloid-producing cell which is common to the pars intermedia and that part of the gland surrounding the upper portion of the stalk. The colloid-producing cell (Fig. 3, *k*) being of least importance may be disposed of first. It is a small cubical or columnar cell found usually forming the epithelial lining of follicles containing transparent colloid material. It has a basal nucleus and mottled, poorly staining protoplasm, relatively poor in mitochondria. The cells usually show little evidence of secretory activity, but occasionally (Fig. 3) one sees in them small droplets of colloid similar to that in the follicles, showing that the follicular colloid is actually a product of these cells.

The other type of cell, the granular cell of the pars intermedia, constitutes the main bulk of this portion of the gland (Fig. 3). It is of a variable prismatic shape conditioned by mutual pressure, and the nucleus is located near one pole of the cell. Alongside of the nucleus or at some distance from it is a mass of deeply staining cytoplasm of about the same size as the nucleus which we presume contains the sphere and centrioles, though we have not actually demonstrated them. This mass is inconspicuous in preparations in which the secretion has

been stained but very obvious in cells from which the secretion antecedents have been removed, or in preparations in which both the secretion and the mitochondria are stained, for as in other cells of this gland the mitochondrion is coextensive as regards distribution with the real cytoplasm of the cell, and its concentrations in the cell indicate the locations of cytoplasmic condensations as distinguished from those parts of the cell where the cytoplasm is dispersed by the masses of secretion. Around this condensation the mitochondria are abundant in the form of short delicate rods. They are also abundant but less so in the perinuclear cytoplasm and they extend radially from the cytoplasmic mass to the periphery of the cell in lines which coincide with the strands of cytoplasm separating secretion-holding spaces. These same strands of cytoplasm are clearly seen in the cells in question from which the secretion has been dissolved as a result of postmortem change or the solvent action of the fixing fluid. In such cells the cytoplasm presents a coarse meshed network which has recently been well illustrated by Rasmussen (1921) in the cells of similar sort from the *pars intermedia* of the woodchuck.

The secretion in these cells is a highly labile material which appears in suitably fixed preparations in the form of small granules which are very difficult to stain. The amount in the individual cell is highly variable, and accordingly in preparations the *pars intermedia* presents a mottled appearance. These granules are not found in the juxtannuclear cytoplasm mass described above. The granules are smaller and stain blue in acid violet but less intensely than those of any of the cells of the anterior lobe. As will be seen later they disappear more rapidly from the cell, as a result of postmortem change, and are less resistant to the solvent action of fixing agents than any of the violet-staining granules of the anterior lobe. In preparations fixed by immersion, frequently only those *pars intermedia* cells which are near the surface of the piece have their secretion granules preserved, those more centrally placed having undergone in various degrees those changes which generally occur in the *pars intermedia* of glands kept several hours before fixation. Even here, however, the *pars intermedia* cell is a distinct secretory type because the granules in the cells of the anterior lobe are little affected.

Postmortem Changes in the Pars Intermedia Cell.

As described under Technique, glands were divided into two equal portions and one was fixed immediately while the other was kept in blood serum for 4 hours, then fixed, and the two lots were compared. In the sections of the piece which had been allowed to undergo this change, no granules were to be found in the cells of the pars intermedia (Fig. 4); instead the cell presented an irregularly vacuolated or rather reticulated appearance due to the withdrawal of the secretion antecedents. This fact possibly explains the variable experimental results obtained by different workers as regards the pressor action of extracts from the pars intermedia and neighboring parts of the hypophysis, for it is probable that so highly soluble a material may diffuse into neighboring sections of the organ to a variable extent, depending on the freshness of the gland and the promptness with which the several parts are separated after removal from the freshly killed animal.

These facts may be summarized as follows: The pars intermedia cells are characterized by their content of secretory granules. These granules are smaller than in any other of the granule-containing cells of the hypophysis. They stain a light shade of blue in the neutral combination of safranin and acid violet, or in the mixture of acid violet and acid fuchsin, or in Mallory's connective tissue stain, after fixation in formalin-Zenker. They are distinguished from all of the chromophil cells of the anterior lobe by the lability of the granules during postmortem change; from both types of blue-staining cells in the anterior lobe by the small size of the granules, and by the fact that the granules are stained lighter and are more diffusely distributed through the protoplasm than the granules of the so called basophils. The pars intermedia cell is distinguished from Type 3 chromophil cell by its lack of vacuoles and its much smaller, lighter stained granules. The cells of the pars intermedia are distinguished from all chromophobes of the anterior lobe by their positive granule content.

The two types of chromophobes are best distinguished from one another by their behavior in sections fixed in strong formaldehyde and formalin-Zenker; in the former large spherical chromophobes are usually abundant.

Study of the Embryonic Hypophysis.

The study of the embryonic hypophysis was undertaken to discover whether there was any relation in time between the appearance of the characteristic pressor effects of the posterior lobe extract and that of the granular secretion antecedents in the cells of the pars intermedia or of hyaline bodies or other structures which have been hypothetically related to the production of the pressor substance.

The technique followed was similar to that employed for the anterior lobe and pars intermedia of the adult.

The cells of the pars intermedia of pig embryos measuring 7.5 cm. in length have large nuclei and very little cytoplasm. The cell boundaries cannot be seen distinctly, and mitoses are abundant. In the 12.5 cm. stage the appearance of the cells is about the same, but vascularization of the mass is indicated by the ingrowth of mesenchyme and blood vessels from the investment of the pars nervosa. The cells are still difficult to delimit. Vascularization is still further advanced in the 17.5 cm. stage, and some of the cells of the deeper layers are increased in size, the increase being due to the expansion of the cytoplasmic body which now begins to show some fine granules (Fig. 5). In some cells these granules are abundant, the cells being fairly packed with them. The granules are minute and have the same staining reactions as the granules of the pars intermedia of the adult. In the later stages a progressively increasing number of cells of the pars intermedia shows this differentiation, and at full term the majority of cells is so differentiated, and the pars intermedia gives the impression of being greater in mass ratio to the other parts of the gland than at any other period of development or growth.

None of the fetal hypophyses contain, so far as we have been able to discover, hyaline bodies.

It is necessary to study serial sections to discover the colloid-containing vesicles, for frequently folds of the pars intermedia, the cavity of which communicates with the residual lumen, present in single sections the appearance of follicles. When actually present the follicles are found to be lined by cylindrical cells with clear non-granular protoplasm and basal nuclei.

These results may now be compared with the physiological action of extracts of the fetal gland as described by Lewis. Lewis found that extracts of the hypophysis of the 7.5 cm. pig were wholly inactive as regards pressor effect even though a very large number of hypophyses was employed in making the extracts. Similarly no definite effects were noted after the injection of extracts of the hypophyses of 12.5 cm. pig fetuses. On the contrary, a marked pressor effect followed injection of extracts made from the hypophyses of 17.5 cm. pigs. The conclusion is obvious—the pressor substance of the posterior lobe of the hypophysis is coincident in time of appearance with the granules or secretory antecedent of the pars intermedia. As an additional reason for assuming a relation between these granules of the pars intermedia cell, it may be pointed out that McCord and Fenger, using the oxytocic method, have found in the new-born calf and lamb that the extracts of the gland were more active per unit weight than in the adult.

CONCLUSIONS.

The bearing of these results on the Herring-Cushing theory of pituitary secretion is apparent. For the first time a true secretion antecedent has been demonstrated in the cells of the pars intermedia, an antecedent which appears in the cells at the same period of development at which active pressor effects may be obtained from the gland extracts. The route of export of this material from the gland to its point of utilization, however, is unknown; it may go by way of the blood or, as required by the Herring theory, by the transneural route to the third ventricle. The objections to the latter conclusion have been amply expanded in the introduction to this paper, but here may be emphasized the fact that the fetal pig hypophysis contains no hyaline bodies. Indeed they are rare in the adult, though there may be seen in the cells of the pars nervosa in the processes of its intrinsic cells, granular deposits which we believe to be the antecedents of the hyaline bodies but which in the pig rarely are discharged and aggregated into discrete masses as in other mammals. The fact that some observers have obtained positive pressor effects from the nervous part of the posterior lobe, exclusive of the pars intermedia, need not weigh very heavily in attempting to trace the course of the secretion,

when we reflect that the difficulties of making such a mechanical separation are almost insuperable, and that the chemical product of the pars intermedia is so soluble and vanishes from the cells so rapidly that it may well be diffusible through the thin membranes which intervene and penetrate post mortem into adjacent parts. We are inclined, therefore, to the view that the secretion leaves the gland by the vascular route rather than by way of the interfibrillar spaces of the pars nervosa.

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EXPLANATION OF PLATE 5.

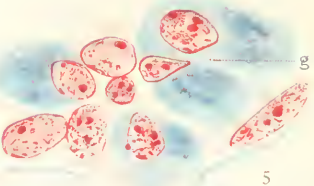
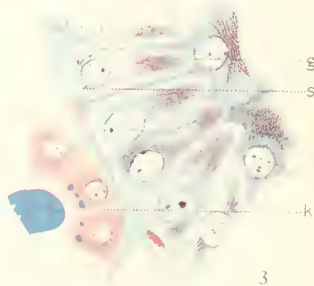
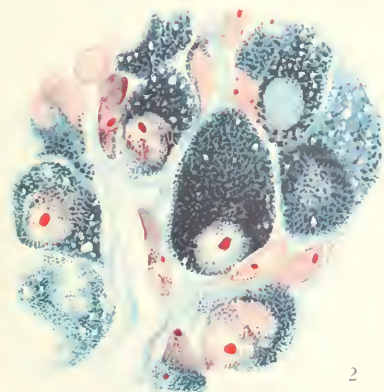
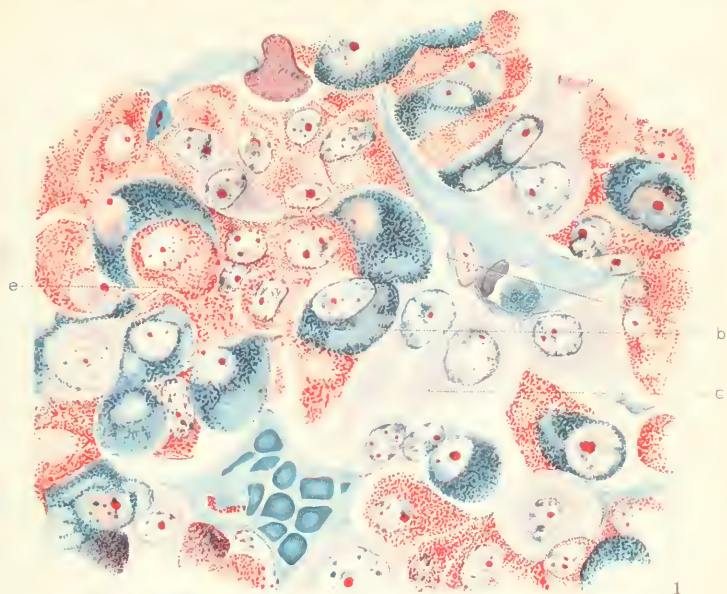
FIG. 1. Anterior lobe of pig hypophysis fixed in formalin-Zenker and stained with safranine-acid violet. (*e*) chromophil cell, Type 1 (eosinophil of authors); (*b*) chromophil cells, Type 2 (basophil of authors); (*c*) chromophobe cell.

FIG. 2. Chromophil cells, Type 3, from the anterior border of the hypophysis of the pig stained with safranine-acid violet.

FIG. 3. Pars intermedia of adult pig hypophysis. Formalin-Zenker; aniline-acid fuchsin and phosphomolybdic water blue. (*k*) part of the wall of a follicle containing colloid, showing colloid cells with colloid droplets at their free borders; (*g*) granular cell of the pars intermedia showing the blue-stained secretion and cytoplasmic mass containing mitochondria; (*s*) granular secretion.

FIG. 4. Two of the chromophobes of the anterior lobe as seen after formalin fixation; acid fuchsin-acid violet stain.

FIG. 5. Group of cells from the pars intermedia of the hypophysis of a fetal pig of 17.5 cm. Four of the cells already have the differentiated characters of the adult pars intermedia. Others are undifferentiated. Formalin-Zenker; acid fuchsin-phosphomolybdic water blue. (*g*) granular secretion antecedent.



THE LOSS OF HEMOLYTIC CAPACITY BY A FRACTION OF A CULTURE OF A HEMOLYTIC STREPTO- COCCUS WITHOUT CHANGE IN AGGLU- TINATION CHARACTERISTICS.

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The partial or complete loss by the hemolytic streptococcus of its ability to hemolyze red blood cells has been recorded. In the studies¹⁻⁴ on variations of streptococci as regards hemolysis and other characteristics, no complete and adequately controlled observations on the agglutination, agglutinin-binding, and agglutinogenic characteristics have been made, as far as we know, to determine whether variation in function has been associated with change in the antigenic constituents of the cocci. We are reporting, therefore, comparative observations on a strain of hemolytic streptococcus which has developed a non-hemolytic fraction.

The strain under consideration is one of the Dochez, Avery, and Lancefield⁵ type strains, No. 32, which had been isolated from the lung at autopsy (bronchopneumonia following measles). This strain was obtained by us during the latter part of 1919 and has been maintained on blood-streaked agar. It was used for the production of immune serum, and also as a control strain in comparative observations on hemolysis. Thus, for more than a year it was plated on pour and

¹ A complete résumé of variations among streptococcus is given by Brown, J. H., The use of blood agar for the study of streptococci, Monograph of The Rockefeller Institute for Medical Research, No. 9, New York, 1919.

² Aschner, P. W., *J. Infect. Dis.*, 1917, xxi, 409.

³ Clawson, B. J., *J. Infect. Dis.*, 1920, xxvii, 368.

⁴ Rosenow, E. C., *J. Infect. Dis.*, 1920, xxvi, 597.

⁵ Dochez, A. R., Avery, T. O., and Lancefield, R. C., *J. Exp. Med.*, 1919, xxx, 179.

streak blood agar plates, at frequent but irregular intervals. During this period no change was observed. When plated in April, 1921, however, it was found that 10 per cent of the colonies showed no evidence of hemolysis but resembled closely a pneumococcus colony. Isolations from both the hemolytic and green types of colonies were made and replated for purity; three substrains of each variety, which for convenience are designated H.1, H.2, H.3 and G.1, G.2, G.3, were used for study.

Up to November 1, 1921, Substrains H.1 and G.1 have been plated thirty times, the others ten times. In no instance has any change been noted in the colony characteristics on blood agar. Since the cleavage was noted, the substrains have bred true for more than 50 transfers.

Cultural Characteristics.

Described according to the recommendations of Smith and Brown⁶ and Brown,¹ the hemolytic fraction has all the characteristics of the beta types with a 4 to 5 mm. transparent zone of hemolysis. The green fraction is an alpha type with no visible clearing except for the late development of a very narrow green translucent zone, which microscopically shows scarcely any diminution in the number of red cells. Immediately surrounding the colony there is no reduction of the number of cells. The surface colonies of the green type fraction are moist, green-zoned, and resemble closely a typical pneumococcus colony.

The hemolytic properties of the two fractions were also tested with the blood tube method (Lyll⁷). With the beta type, hemolysis was prompt; 0.3 cc. of a 15 hour broth culture caused complete hemolysis, and as little as 0.05 cc. caused partial hemolysis. The green fraction showed no hemolysis with 0.5 cc.; the color of the red cells was changed to a deep magenta, but not to the typical brown shown by the pneumococcus used as a control.

Morphologically and culturally the two fractions show no differences, other than those noted above. Planted in 1 per cent carbo-

⁶ Smith, T., and Brown, J. H., *J. Med. Research*, 1914-15, xxxi, 455.

⁷ Lyll, H. W., *J. Med. Research*, 1914, xxx, 515.

hydrate media (glucose, lactose, salicin, mannitol, raffinose, and inulin) all fishings have shown the same end-reaction. This was tested by the colorimetric method to determine the final H ion concentration. Neither fraction is soluble in bile.

Agglutination and Agglutinin Absorption Tests.

Agglutination tests were set up with the serum of a sheep immunized against the original Strain 32. Fortunately all fishings of the hemolytic and green fractions grew with a diffuse cloud in beef broth so there was no difficulty in obtaining satisfactory agglutination results. The results of the test with the sheep serum showed that both fractions were agglutinated equally well, and to the titer of the serum strain, No. 32. Likewise the absorption of this serum by either fraction removed completely the agglutinins for both fractions and for the serum strain, No. 32.

For more conclusive evidence rabbits were immunized with a representative of each of the fractions. At each injection control plates were made to check the character of the injected cocci. The serums obtained were used for agglutination and agglutinin absorption tests. The results are given in Table I.

Direct agglutination and agglutinin absorption tests, with the antisera for both the hemolytic and green fractions, were also carried out on the original Strain 32, and on the other fishings, H. 2, H. 3 and G. 2, G. 3, isolated at the time the cleavage was noted. The reactions of these strains were identical with those of the serum strains H. 1 and G. 1 given in Table I.

The ability of either fraction to absorb the agglutinins from the antiserum of the other fraction shows that the loss of hemolytic power has not been accompanied by any change in the agglutinogenic complex of the cocci.

Virulence Tests.

The virulence of the two strains was tested by mouse inoculation with 18 hour broth cultures. With the hemolytic variety 0.5 cc. killed uniformly, 0.25 cc. irregularly. The green type was less virulent, 1 to 1.5 cc. causing death, but only irregularly.

TABLE I.

Agglutination and Agglutinin Absorption Tests on the Hemolytic and Non-Hemolytic Fractions of Strain 32.

Antiserum.	Absorbed by substrain.	Absorbing dose.	Agglutination.						
			Substrain.	1:50	1:100	1:400*	1:2,000*	1:5,000	Broth.
H. 1†	0‡	0‡	H. 1†	++	++	++	++	+	—
			G. 1§	++	++	++	++	±	—
	H. 1	1:20	H. 1	—	—	—	—	—	—
			G. 1	—	—	—	—	—	—
	G. 1	1:20	H. 1	—	—	—	—	—	—
			G. 1	—	—	—	—	—	—
	H. 1	1:50	H. 1	+	×	—	—	—	—
			G. 1	±	×	—	—	—	—
	G. 1	1:50	H. 1	+	×	—	—	—	—
			G. 1	±	×	—	—	—	—
G. 1	0‡	0‡	H. 1	++	++	++	++	+1	—
			G. 1	++	++	++	++	+	—
	H. 1	1:20	H. 1	—	—	—	—	—	—
			G. 1	—	—	—	—	—	—
	G. 1	1:20	H. 1	—	—	—	—	—	—
			G. 1	—	—	—	—	—	—
	H. 1	1:50	H. 1	+	±	—	—	—	—
			G. 1	±	×	—	—	—	—
	G. 1	1:50	H. 1	+	±	—	—	—	—
			G. 1	±	±	—	—	—	—

Normal serums of the rabbits obtained before immunization did not agglutinate any of the strains.

Heterologous cultures of hemolytic and non-hemolytic streptococci were used as negative absorption controls. In a dose of 1:10 they did not reduce the specific titer of the serums.

++ indicates complete agglutination; +1, +, ±, ×, decreasing amount; —, no agglutination.

* To save space intermediate dilutions set up with unabsorbed and absorbed serums are not listed.

† H. 1 indicates hemolytic fraction.

‡0 indicates that the serum was not absorbed.

§ G. 1 indicates non-hemolytic or green fraction.

|| Absorbing dose 1:20 or 1:50 means that the mass of cocci is to the volume of serum (diluted 1:15) as 1 is to 20 or 1 is to 50.

Attempts were made to increase the virulence of the green variant by mouse passage in the hope that the hemolytic capacity might return with the increase in virulence. After fourteen consecutive passages the virulence was raised so that $\frac{1}{60}$ cc. caused death. This increased virulence was not associated with the development of hemolytic colonies. The mouse passage caused no marked fall in agglutinability and no change in the absorptive capacity of the culture. A further attempt to restore the hemolytic capacity to the green variant was made by growing it in blood broth, but twenty-five successive transfers in this medium have resulted in no change. The hemolytic fraction has also bred true after twenty-five blood broth generations.

Replating of the original Strain 32 showed the persistence of the two varieties throughout August, 1921. At that time fresh isolations of the two fractions were made and found to be identical culturally and serologically with the previous isolations. Since then, two platings of the original strain have failed to show any green colonies although a large number of plates was made.

Tests with Other Strains.

Twenty other strains of hemolytic streptococcus, the majority from pathological conditions, which had been under cultivation for from 2 to 8 years, were tested in order to determine whether or not any loss of hemolysis had occurred. Frequent platings of these strains have shown no change in the hemolytic capacity, as all colonies give well marked zones. These observations are in contrast with those of Clawson³ who found that out of 116 strains of hemolytic streptococcus under cultivation for 1 to 4 years, 54 showed one or more green colonies when plated in blood agar. He tested the constancy of substrains obtained from six cultures. His substrains showed little tendency to breed true, the green substrains in all instances showing reversion to the hemolytic type.

DISCUSSION.

Further observations on the antigenic character of bacterial strains in relation to functional change are needed for reconsideration of the question of so called bacterial mutation. The example which we are

reporting of an apparently sudden and permanent functional loss, that is of hemolytic capacity by a portion of a strain without associated antigenic change, is presented for this reason.

Variations in the physiological functions of bacteria occur with frequency. This has given rise to an impression held by some that the fundamental antigenic constituents are also readily subject to change. As a matter of fact, general experience indicates a high degree of stability of bacterial types as regards the antigenic qualities of their body substance. In the light of this experience it would seem more logical to assume the sceptical attitude that a change in the antigenic constituents is a fundamental and radical change, and, therefore, an unusual occurrence. The proof adduced for such a change, therefore, must be proportionately complete and convincing.

The proof must be complete in two ways. First, is the variant real or is it a contaminant of the original culture, or a contaminant added during transfer or by animal passage, etc.? Second, does the evidence advanced in favor of antigenic change prove it to be an actual basic change or simply show that a modification of the binding or reacting characteristics has occurred? The evidence for a basic change in the antigenic constituents must include a study of the agglutinogenic capacities, as well as of the reacting and combining capacities of both the variant and unchanged fraction.

CONCLUSIONS.

A culture of hemolytic streptococcus developed a non-hemolytic fraction which as determined by its agglutinogenic, agglutination, and agglutinin absorption characters was identical with the hemolytic fraction, the only variation being one of function, or physiological action. This functional variation has proved permanent, as long as observed, for both fractions have continued to breed true.

This study is offered as additional evidence in favor of the hypothesis that functional changes among bacteria are, at most, only very infrequently associated with changes in the antigenic matrix of bacteria.

THE ORGANOTROPIC, BACTERIOTROPIC, AND LEUCOCYTOTROPIC ACTIONS OF CERTAIN ORGANIC CHEMICALS.

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Present knowledge of the manner by which chemicals influence the mechanism of infection and resistance is limited. Investigations in chemotherapy hitherto have been concerned primarily with certain organotropic and bacteriotropic activities,—a search for a chemical of a monotropic character. The influence of definite chemical entities both upon the factors vital to the integrity of the animal against an invading microorganism, and such life processes as are perhaps not essential to actual defense against bacterial disease, has not been thoroughly investigated. The work of Ehrlich, Morgenroth, Browning, and others has demonstrated that it is possible to find chemicals with less of organotropic than of bacteriotropic activity. Frequently, however, when such substances have been used against an experimental infection, the treated animal succumbed as though no drug had been given, or in a shorter time than the controls. In other words, the drug, in doses not fatal under normal conditions, apparently acts upon defensive complexes necessary to the animal in coping with an infection, having a greater influence upon this activity than upon the pathogenic microorganism. Just what the force or combination of forces is that maintains a state of stable resistance in the animal toward microorganisms can only be conjectured. Whatever other elements enter into this biological reaction, obviously the phagocyte has some share. Because of this, as evidenced in the increase of the activity of the leucocyte during the course of many infectious diseases, it was decided to study the influence of certain chemicals, not alone in respect to organotropism and to bacteriotropism, but as to leucocyto-

tropism as well. The work was undertaken in the hope that information might be gained which would enable a more intelligent choice of chemicals for chemotherapeutic purposes, and might lead to the finding of a delicate indicator by which to judge the relationship between chemical constitution and the activity exhibited by the body against an invading organism.

We wish to report the toxicity for mice (organotropism), the bactericidal action on *Staphylococcus aureus* (bacteriotropism), and the antiphagocytic influence (leucocytotropism), of certain members of seven groups of chemicals—triphenylmethane leuco bases, triphenylmethane dyes, acridines, safranines, phenazines, quinones, and cinchonas.

The work was done while in collaboration with Dr. Jacobs and Dr. Heidelberger in the study of chemotherapy.¹ The manner in which the chemicals here reported influence an experimental pneumococcus infection of mice will appear in a later publication.

The confusion that exists in the literature on phagocytosis seems to be due for the most part to variations in technique. Even in controlled phagocytic studies frequent anomalies appear, unavoidable when dealing with so delicate an indicator as the leucocyte. If, then, instead of considering results from one such experiment or series of experiments, we attempt to compare those from work done under widely differing conditions, definite conclusions are difficult to reach.

The findings of Hamburger,² Kolmer,³ Manwaring and Ruh,⁴ Grünspan,⁵ and Smith⁶ on the influence of quinine on phagocytosis can hardly be compared. Hamburger⁷ obtained leucocytes from defibrinated horse blood and suspended them in horse serum. To this suspension he added the drug and incubated the mixture, thus affording opportunity for interaction between quinine and leucocytes

¹ Felton, L. D., and Dougherty, K. M., *J. Exp. Med.*, 1922, xxxv, 761.

² Hamburger, H. J., *Centr. Bakt., 1te Abt., Ref.*, 1913, lvii, 105. Hamburger, H. J., and Hekma, E., *Biochem. Z.*, 1908, ix, 512.

³ Kolmer, J. A., Solis-Cohen, S., and Steinfield, E., *J. Infect. Dis.*, 1917, xx, 333.

⁴ Manwaring, W. H., and Ruh, H. O., *J. Exp. Med.*, 1907, ix, 473.

⁵ Grünspan, T., *Centr. Bakt., 1te Abt., Orig.*, 1909, xlviii, 444.

⁶ Smith, H. L., *Lancet*, 1910, ii, 1342.

⁷ Hamburger, H. J., and Hekma, E., *Biochem. Z.*, 1907, iii, 88.

before the charcoal was added. Kolmer,³ on the other hand, used washed leucocytes procured from the peritoneal cavity of rabbits after an injection of aleuronat, and these came into contact with the quinine only after it had been incubated an hour with a heavy suspension of living Type I pneumococci. Thus in the former case, the quinine acted primarily on the leucocyte and secondarily on the substance to be phagocytosed, while in the latter, the primary action was on the pneumococcus. As might be expected, their results contradict each other, Hamburger² claiming an inhibition in phagocytic activity with a dilution of quinine 1:1,000, and Kolmer³ claiming a stimulation with the same strength of the drug. Manwaring and Ruh,⁴ using still another technique, found a 20 per cent stimulation from quinine in as low a dilution as 1:200. In this instance whole defibrinated blood was used with a streptococcus which had been suspended in 0.85 per cent NaCl and sterilized at 100° C. Grünspar⁵ also places the optimum concentration for this drug at 1:200. Smith⁶ gives 1:7,500 as the lowest dilution of quinine stimulating phagocytosis. He used washed human corpuscles, human serum, and living *B. coli*, sealing them in a Wright tube and incubating in an opsonizer. The fact that the chamber was sealed may have exerted an influence on the process.

Methods.

Although present knowledge relating to phagocytosis does not make it possible to employ a method which will give uniformly constant results, by testing the chemicals in groups, each group on the same day, with the same leucocytes, staphylococci, and guinea pig serum, we have endeavored to render the conditions of our experiments constant.

The following three methods for determining the degree of phagocytosis were tried.

1. 0.5 cc. of leucocytic suspension + 0.5 cc. of culture + 0.5 cc. of guinea pig serum 1:10 + 0.5 cc. of chemical were mixed in a 12 mm. tube and incubated for 15 minutes in a water bath at 37.5°C.

2. 0.5 cc. of leucocytic suspension + 0.5 cc. of chemical were mixed and incubated as above for 10 minutes. To this were then added 0.5 cc. of culture and 0.5 cc. of guinea pig serum 1:10, the mixture was shaken, and the tube was reincubated for 10 minutes in a water bath at 37.5°C.

3. 0.5 cc. of leucocytes suspended in undiluted guinea pig serum + 0.5 cc. of culture + 0.5 cc. of chemical were incubated for 15 minutes in a water bath at 37.5°C.

The last was the method finally adopted, since the simultaneous incubation of all constituents mixed together seemed more nearly to reproduce actual conditions of infection in the animal.

The leucocytes were procured from the peritoneal cavity of a guinea pig 15 hours after an intraperitoneal injection of a solution composed of 3 per cent aleuronat, 6 per cent starch, in 0.85 per cent NaCl, by washing out the exudate with sterile 0.5 per cent citrate in 0.85 per cent NaCl. The cells were washed three times in sterile citrate solution before being used. Opsonin and complement were furnished by fresh guinea pig serum and the organism employed as the indicator of leucocytic activity was an 18 hour broth culture of *Staphylococcus aureus*.

Smears were made after incubation, and the preparations stained by Cross's⁸ method. The staphylococci contained in 100 cells were counted and the counts averaged. The result of each dilution of the chemical is shown in Tables I to VIII, the dilution quoted being calculated after the addition of all the components. Uniform dilutions of the different chemicals could not be employed, due to the varying solubilities of the drugs. Control counts were made from tubes containing leucocytes, serum, and organisms, but no chemical.

The toxicity given is the largest, non-fatal intraperitoneal dose in milligrams for 18 gm. mice.

The bactericidal action of the drugs was determined in whole blood with a 2 hour incubation period, by means of a technique described in a previous paper.¹

Triphenylmethane Leuco Bases.

As a group, the triphenylmethane leuco bases studied inhibit phagocytosis (Table I), only 33 per cent of the total number of compounds showing a normal count in the highest dilution used. In analyzing this inhibition, consistent correlation with bactericidal potency and toxicity is hard to follow.

For instance, leucomalachite green (D 1) and leucobenzein (D 8) have the same strength as regards bactericidal action, killing *Staphylococcus aureus* at 1:400. But D 1, causing a 60 per cent reduction of the phagocytic index at 1:40,000, permits an approximately normal count at 1:160,000, while D 8 at 1:600,000 shows an 80 per cent inhibition of this activity. The maximum non-lethal dose for D 1

⁸ Cross, H. B., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 350.

is 1.0 mg., for D 8, 2.5 mg., so that D 1, while two and one-half times as toxic and having the same bactericidal action, allows a much greater degree of phagocytosis than D 8.

p-Hydroxyleucomalachite green (D 2) and *p*-aminoleucomalachite green (D 3) show divergence in another direction. Both have a maximum non-lethal dose of 1.0 mg. D 3 exerts no bactericidal power at 1:400, while D 2 kills at that dilution. At 1:160,000, D 2 exhibits an approximately normal index, D 3 a 33 per cent reduction. At 1:320,000, D 2 shows a 50 per cent reduction and D 3 only a 25 per cent.

A third variation in effect is seen between *o*-hydroxyleucomalachite green (D 9) and *o*-methoxyleucomalachite green (D 11). The phagocytic indices are reduced 80 per cent by both chemicals at 1:320,000, but neither has any bactericidal power at 1:400, and their maximum non-lethal doses differ widely, D 9 being two and one-half times as toxic as D 11.

Triphenylmethane Dyes.

In comparing the specific dyes with their leuco bases the same irregularity is found. Neither *o*-hydroxymalachite green (D 7) nor its leuco base (D 9) in 1:400 possesses bactericidal power; D 9, however, is two and one-half times as toxic, and at 1:600,000 reduces the phagocytic index 90 per cent as compared with 25 per cent by D 7 in the same dilution (Table II).

In the case of the *p*-methoxymalachite green (D 12) and its leuco base (D 10), the dye is more toxic and bactericidal than the leuco base, but from 1:15,000 to 1:1,500,000, D 12 shows a normal phagocytic index, while at 1:600,000, D 10 reduces it 66 per cent. It may be noted here that *p*-methoxymalachite green (D 12) and ethylviolet chloride (D 32) are exceptional in allowing normal indices in a bactericidal dilution.

TABLE I.
Triphenylmethane Leuco Bases.

Chemical.	Maximum non-lethal dose intraperitoneally.		Bactericidal for staphylococci in whole blood in 2 hrs.	Effect on phagocytic index of different dilutions of chemicals.				
	Mg.	Dilution in mouse.		1:40,000	1:80,000	1:160,000	1:320,000	Control.
(D 1) Leucomalachite green.	1.0	1:3,000	0 at 1:400	3.5	3.8	8.6	11.0	9.9
(D 3) <i>p</i> -Aminoleucomalachite green.	1.0	1:3,000	∞ " 1:400	3.2	4.1	6.0	7.1	9.9
(D 6) <i>p</i> -Methylleucomalachite green.	2.5	1:1,200		4.4	4.0	7.3	4.4	9.9
				1:1,500	1:15,000	1:150,000	1:1,500,000	Control.
(D 13) <i>p</i> -Ethoxyleucomalachite green.	0.5	1:6,000	∞ at 1:400	3.6	5.0	5.0	6.2	6.0
				1:40,000	1:80,000	1:160,000	1:320,000	Control.
(D 2) <i>p</i> -Hydroxyleucomalachite green.	1.0	1:3,000	+ at 1:400	3.2	5.9	8.8	6.4	9.9
				1:600	1:6,000	1:60,000	1:600,000	Control.
(D 9) <i>o</i> -Hydroxyleucomalachite green.	0.5	1:6,000	∞ at 1:400	2.4	2.7	2.6	2.5	12.3
				1:1,500	1:15,000	1:150,000	1:1,500,000	Control.
(D 14) 2,4-Dihydroxyleucomalachite green.	1.25	1:2,400	∞ at 1:400	2.8	3.9	6.6	4.3	6.4
				1:600	1:6,000	1:60,000	1:600,000	Control.
(D 11) <i>o</i> -Methoxyleucomalachite green.	1.25	1:2,400	∞ at 1:400	1.2	2.3	2.1	2.5	12.3

TABLE I—*Concluded.*

Chemical.	Maximum non-lethal dose intraperitoneally.		Bactericidal for staphylococci in whole blood in 2 hrs.	Effect on phagocytic index of different dilutions of chemicals.				
	Mg.	Dilution in mouse.		1:40,000	1:80,000	1:160,000	1:320,000	Control.
(D 4) Leucocrystal violet.	1.0	1:3,000	∞ at 1:350	2.4	1.2	4.4	3.4	9.9
				1:600	1:6,000	1:60,000	1:600,000	Control.
(D 10) <i>p</i> -Methoxyleucomalachite green.	2.5	1:1,200	0 at 1:400	4.5	3.2	2.4	4.2	12.3
(D 8) Leucobenzein.	2.5	1:1,200	0 " 1:400		3.2	2.6	2.0	12.3

The blanks in the tables indicate that the cells in those dilutions were too disintegrated to count.

In general, the leuco compounds are less bactericidal than the dyes, the difference being quite marked in some cases.

- (D 18) Malachite green nitrate..... 1 : 6,400
 (D 1) Leucomalachite green..... > 1 : 400
 (D 16) *p*-ethoxymalachite green chloride..... 1 : 12,800
 (D 13) *p*-ethoxyleucomalachite green chloride..... > 1 : 400
 (D 15) *o*-methoxymalachite green nitrate..... 1 : 6,400
 (D 11) *o*-methoxyleucomalachite green nitrate..... > 1 : 400

This bactericidal relationship between the dye and its corresponding leuco base is not found in regard to the antiphagocytic action. Without exception both leuco bases and dyes decrease the phagocytic index, and the dilution represented by the largest non-lethal dose in the mouse is more leucocytotropic than bacteriotropic.

Acridines.

The acridines represented in Table III yield findings very similar to that with the triphenylmethane dyes, although as a group they are not so bactericidal, nor is perhaps the antiphagocytic action so great. In comparing the chemicals of this group, proflavine (D 26) stands out as possessing almost ideal characteristics, very similarly to *p*-methoxymalachite green (D 12) and ethyl violet (D 32) of the triphenylmethane dyes; the tropic relationships of the chemicals are such that a dose non-lethal for mice is also bactericidal for staphylo-

TABLE II.
Triphenylmethane Dyes.

Chemical.	Maximum non-lethal dose intraperitoneally.		Bactericidal for staphylococci in whole blood in 2 hrs.	Effect on phagocytic index of different dilutions of chemicals.				
	Mg.	Dilution in mouse.		1:600	1:6,000	1:60,000	1:600,000	Control.
(D 18) Malachite green nitrate.	0.6	1:5,000	0 at 1:6,400	0.24	—	11.5	15.0	14.1
				1:1,200	1:12,000	1:120,000	1:1,200,000	Control.
(D 16) <i>p</i> -Ethoxymalachite green chloride.	0.6	1:5,000	0 at 1:12,800	—	1.9	2.8	—	4.2
(D 19) <i>p</i> -Hydroxymalachite green chloride.	0.3	1:9,000	∞ " 1:1,600	—	—	1.8	2.3	4.0
				1:600	1:6,000	1:60,000	1:600,000	Control.
(D 7) <i>o</i> -Hydroxymalachite green.	1.25	1:2,400	∞ at 1:400	1.8	2.1	3.0	3.0	4.1
				1:1,500	1:15,000	1:150,000	1:1,500,000	Control.
(D 15) <i>o</i> -Methoxymalachite green nitrate.	0.6	1:5,000	0 at 1:6,400	4.1	4.4	6.8	5.0	6.4
				1:40,000	1:80,000	1:160,000	1:1,320,000	Control.
(D 5) Hexamethylviolet (crystal).	1.0	1:3,000	0 at 1:350 24 hrs. 0 at 1:39,400	0.0	3.0	8.8	4.4	7.4
				1:900	1:9,000	1:90,000	1:900,000	Control.
(D 20) <i>p</i> -Tolylmalachite green chloride.	0.03	1:90,000	0 at 1:2,000	—	—	—	—	9.1

TABLE II—*Concluded.*

Chemical.	Maximum non-lethal dose intraperitoneally.		Bactericidal for staphylococci in whole blood in 2 hrs.	Effect on phagocytic index of different dilutions of chemicals.				
	Mg.	Dilution in mouse.		1:1,200	1:12,000	1:120,000	1:1,200,000	Control.
(D 17) <i>o</i> -Chloromalachite green chloride.	0.6	1:5,000	0 at 1:1,600	—	0.8	3.4	2.3	3.4
				1:600	1:6,000	1:60,000	1:600,000	Control.
(D 24) <i>p</i> -Chloromalachite green nitrate + 3.5 H ₂ O.	0.5	1:6,000	+ at 1:2,500	—	1.3	2.2	3.2	7.1
(D 21) Brilliant green nitrate + 1 H ₂ O.	0.003	1:900,000	0 " 1:4,000	3.1	5.0	3.3	7.9	7.8
(D 32) Ethylviolet chloride	0.125	1:24,000	0 " 1:8,000	4.5	13.0	12.0	13.0	12.3
(D 31) 3, 4-Methylene-dihydroxymalachite green chloride + 4 H ₂ O.	0.06	1:50,000	0 " 1:8,000	6.8	11.0	7.0	12.5	12.3
(D 27 a) 2-Ethoxy-4', 4''-bisdimethylaminotriphenylcarbinol.	0.03	1:90,000	0 " 1:500	—	1.0	—	9.0	14.1
(D 27 b) <i>o</i> -Ethoxymalachite green.	1.25	1:2,400	0 " 1:500	—	—	—	—	14.1
(D 22) <i>p</i> -Nitromalachite green chloride.	0.03	1:90,000	+ " 1:2,000	—	1.3	5.5	4.8	7.1
				1:1,500	1:15,000	1:150,000	1:1,500,000	Control.
(D 12) <i>p</i> -Methoxymalachite green.	0.5	1:6,000	0 at 1:3,200	4.5	8.0	7.5	8.5	7.6

coccus and in this concentration not antiphagocytic. Since the introduction of acriflavine by Ehrlich⁹ members of this group of chemicals have been employed in the treatment of various infections.

Browning and Cohen¹⁰ have recently reported on the antiseptic properties of a rather complete series of acridines, and although not all derivatives were the same

⁹ Ehrlich, P., and Benda, L., *Ber. chem. Ges.*, 1913, xvi, 1931.

¹⁰ Browning, C. H., and Cohen, J. B., *Brit. Med. J.*, 1921, ii, 695.

TABLE III.

Acridines.

Chemical.	Maximum non-lethal dose intraperitoneally.		Bactericidal for staphylococci in whole blood in 2 hrs.	Effect on phagocytic index of different dilutions of chemicals.				
	Mg.	Dilution in mouse.		1:600	1:6,000	1:60,000	1:600,000	Control.
(D 54) 3, 6-Dihydroxy-acridine.	1.25	1:2,400	∞ at 1:1,000	0.5	5.2	7.9	15.0	10.3
(D 53) 3, 6-Diamino-9-phenylacridine.	1.25	1:2,400	∞ " 1:1,000	—	1.3	0.5	1.6	10.3
(D 60) Diamino- <i>n</i> -methyl-acridone.	1.25	1:2,400	∞ " 1:500	2.5	4.5	11.3	7.1	15.1
(D 43) 9, 10-Dimethyl-acridinium chloride.	0.625	1:5,000	∞ " 1:500	—	3.0	6.1	6.1	12.3
				1:200	1:2,000	1:20,000	1:200,000	Control.
(D 34) 3, 6-Diamino-10-methylacridinium chloride + 1 H ₂ O.	0.5	1:6,000	0 at 1:1,000	0.8	3.9	7.2	6.2	12.3
				1:600	1:6,000	1:60,000	1:600,000	Control.
(D 57) 2-Amino-6-hydroxy-acridine dihydrochloride.	2.5	1:1,200	∞ at 1:500	—	3.0	1.2	1.2	10.3
(D 26) Diaminoacridine sulfate + 3 H ₂ O.	2.5	1:1,200	0 " 1:500	1.7	17.8	14.0	13.0	14.1
(D 49) Leucocyanotrypaflavine dihydrochloride.	0.312	1:9,000	+ " 1:500	1.8	5.6	9.2	9.6	10.3
(D 42) Cyanotrypaflavine.	0.078	1:38,400	0 " 1:500	4.0	6.4	2.6	8.7	12.3
(D 56) Acridine orange dihydrobromide.	0.625	1:5,000	∞ " 1:500	—	—	1.9	3.2	10.3
(D 40) Homoflavine ("acridine yellow").	0.6	1:5,000	∞ " 1:1,000	—	4.5	5.5	7.8	12.3

as those given here, confirmation was made in respect to bactericidal action on staphylococcus. These authors did not study the influence of the group on the phagocytic index. Gay and Morrison¹¹ using acriflavine claim negative chemotherapeutic results in experimental streptococcus empyema in rabbits, regardless of

¹¹ Gay, F. P., and Morrison, L. F., *J. Infect. Dis.*, 1921, xxviii, 1.

the high bactericidal potency of the drug against this organism. They show phagocytosis to be inhibited by strong concentrations, such as have usually been employed, but state that the dye sterilizes considerable quantities of pus in the test-tube in a dose which does not inhibit phagocytosis.

Browning, Gulbransen, Kennaway, and Thornton¹² report that acriflavine kills staphylococcus in a dilution of 1:100,000 in serum and does not inhibit phagocytosis above 1:500. It is difficult to compare our results with theirs, as a different medium was used, the incubation period was shorter in performing the bactericidal test, they did not consider phagocytosis inhibited unless the inhibi-

TABLE IV.

Safranines.

Chemical.	Maximum non-lethal dose intraperitoneally.		Bactericidal for staphylococci in whole blood in 2 hrs.	Effect on phagocytic index of different dilutions of chemicals.				
	Mg.	Dilution in mouse.		1:600	1:6,000	1:60,000	1:600,000	Control.
(D 63) Methyl-naphthophenazonium chloride.	0.156	1:19,200	+ at 1:500	—	—	9.7	8.6	15.1
(D 62) Isorosindulin nitrate.	2.5	1:1,200	+ " 1:500	—	2.4	—	—	15.1
(D 37) Phenosafranine chloride.	0.078	1:38,400	+ " 1:500	1.8	8.5	7.1	9.0	12.3
				1:200	1:2,000	1:20,000	1:200,000	Control.
(D 47) Phenylrosindulin chloride.	0.625	1:4,800	0 at 1:500	0.8	1.3	2.8	2.1	12.3

tion exceeded 50 per cent, and they used human materials in their method. Despite the toxic action of proflavine and acriflavine for the phagocyte, they seem to have some local therapeutic action. According to Davis¹³ both of these chemicals, following either an intravenous injection or *per os* administration, render the urine bactericidal for both staphylococcus and the colon bacillus. Davis and Harrell¹⁴ also report a therapeutic action of acriflavine in treatment of gonorrheal urethritis.

Safranines.

The small number of safranines studied does not warrant any general deductions as to this group of compounds. But for the staphylo-

¹² Browning, C. H., Gulbransen, R., Kennaway, E. L., and Thornton, L. H. D., *Brit. Med. J.*, 1917, i, 73.

¹³ Davis, E. G., and Beck, G. H., *J. Urol.*, 1921, v, 215.

¹⁴ Davis, E. G., and Harrell, B. E., *J. Urol.*, 1918, ii, 257.

coccus, the chemicals shown in Table IV exert a very low bactericidal action. And they are markedly antiphagocytic with the exception of phenosafranine, which produces a 25 per cent reduction in the phagocytic index in a dilution of 1:600,000.

TABLE V.

Phenazines.

Chemical.	Maximum non-lethal dose intraperitoneally.		Bactericidal for staphylococci in whole blood in 2 hrs.	Effect on phagocytic index of different dilutions of chemicals.				
	Mg.	Dilution in mouse.		1: 600	1: 6,000	1: 60,000	1: 600,000	Control.
(D 65) <i>unsym.</i> -Diaminophenazine hydrochloride.	1.25	1:2,400	∞ at 1:500	—	6.3	5.6	10.6	15.5
(D 38) <i>unsym.</i> -Diaminosafrafranine hydrochloride.	2.5	1:1,200	∞ " 1:500	—	2.8	6.5	8.9	12.3
				1: 200	1: 2,000	1: 20,000	1: 200,000	Control.
(D 46) <i>sym.</i> -Diaminophenazine hydrochloride.	1.25	1:2,400	+ at 1:500	—	2.8	2.9	4.0	12.3
				1: 600	1: 6,000	1: 60,000	1: 600,000	Control.
(D 36) Toluylene red hydrochloride.	1.25	1:2,400	∞ at 1:500	3.2	6.0	3.3	8.4	12.3
				1: 200	1: 2,000	1: 20,000	1: 200,000	Control.
(D 44) Dimethylnaphthoenrhodine hydrochloride.	0.6	1:5,000	∞ at 1:500	—	3.5	6.5	5.0	12.3

Phenazines and Quinones.

The phenazines (Table V), and quinones (Table VI) both may be classed as drugs that are not bactericidal for staphylococci, but they are antiphagocytic in dilutions non-toxic for a mouse, with the exception of sodium chloranilate in the quinone group. This compound apparently has no bactericidal action, yet permits of phagocytosis within 25 per cent of normal in a dilution of 1:600.

TABLE VI.

Quinones.

Chemical.	Maximum non-lethal dose intraperitoneally.		Bactericidal for staphylococci in whole blood in 2 hrs.	Effect on phagocytic index of different dilutions of chemicals.				
	Mg.	Dilution in mouse.		1:600	1:6,000	1:60,000	1:600,000	Control.
(D 58) Quinone.	0.625	1:5,000	∞ at 1:1,000	4.0	2.3	1.0	4.2	10.3
(D 59) <i>p</i> -Nitrosophenol.	0.312	1:9,000	+ " 1:50	2.3	3.2	5.6	13.0	15.5
(D 64) Sodium chloranilate.	5.0	1:600	∞ " 1:500	11.1	14.2	14.2	2.0	15.1
(D 67) Anilino- β -naphtha quinone.	1.25	1:2,400	∞ " 1:1,000	—	6.0	12.0	7.7	10.3

Cinchonas.

The cinchona compounds in Table VII are the members of a group of alkaloids on which we reported in a previous paper.¹ It was found that the aromatic compounds had the power of killing, rapidly, multiple lethal doses of virulent pneumococci when organisms and drugs were injected simultaneously into the peritoneal cavity of mice. In this respect, all cinchona derivatives were found to be superior to optochin, but the different aromatic substitution products were possessed of varying degrees of bactericidal action *in vitro* and *in vivo*. Intravenous treatment with these drugs lowered the resistance of the mice as did any method of treatment except *per os*, when given at a site other than the one at which the organisms had been injected. Although mice with definitely established pneumococcus infection were not cured, these compounds, under specified conditions, had a measurable amount of protective action, greater perhaps than that of any drug used heretofore against a bacterial infection.

At a glance the compounds in therapeutic doses in Table VII are seen to possess marked antiphagocytic activity, and with the possible exception of C 29, the aromatic compounds are seen to be more leucocytotropic than optochin. The question naturally arises, whether this antiphagocytic property is the cause of the therapeutic failure of the chemicals in an established infection.

TABLE VII.

Cinchonas.

Chemical.	Maximum non-lethal dose intraperitoneally.		Bactericidal for staphylococci in whole blood in 2 hrs.	Effect on phagocytic index of different dilutions of chemicals.			
	Mg.	Dilution in mouse.		1:1,000	1:2,000	1:4,000	Control.
(C 29) Hydroquinine chloroacetyl-anilide.	0.5	1:6,000		—	6.0	3.7	7.7
(C 36) Dihydroquinine <i>p</i> -chloro-acetylaminophenol hydrochloride.	2.5	1:1,200	+ at 1:1,000	—	—	2.4	7.7
(C 40) Dihydroquinine <i>m</i> -chloro-acetylaminophenol hydrochloride.	1.5	1:1,900	+ “ 1:2,000	1.9	3.2	∞	7.7
(C 110) Dihydroquinine 4-chloro-acetylaminopyrocatechol hydrochloride.	4.0	1:750	+ “ 1:500	—	1.2	0.72	7.7
(C 11) Optochin (ethylhydrocupreine).	3.0	1:960	+ “ 1:500	5.2	1.8	3.36	7.7
(C 9) Hydroquinine.	1.0	1:3,000		4.6	6.0	4.0	7.7

Bordet's experiments¹⁵ showing that the resistance of an animal is lowered after it has been partially depleted of leucocytes by means of carmine, and also Lippman's work¹⁶ proving that optochin has no protective action in animals whose leucocyte count has been lowered by thorium, may be cited in support of the postulate that a drug which inhibits the action of the leucocytes will have little value in treatment of a bacterial disease. However, the experiments of neither Bordet¹⁵ nor Lippman¹⁶ are conclusive for the reason that the chemicals used to decrease the number of white cells may have exerted a toxic action on other functions vital to the resistance of the animal. Acton¹⁷ has demonstrated that a number of cinchona derivatives inhibit completely the migration of leucocytes in a dilution of 1:500. The classical work of Binz¹⁸ shows that quinine itself, along with its other general protoplasmic toxicities, inhibits phagocytosis.

¹⁵ Bordet, J., *Studies in immunity*, translated by Gay, F. P., New York, 1909, 30.

¹⁶ Lippman, *Z. Immunitätsforsch., Orig.*, 1915-16, xxiv, 107.

¹⁷ Acton, H. W., *Lancet*, 1922, i, 124.

¹⁸ Binz, C., *Das Chinin. Nach den neuern pharmakologischen Arbeiten dargestellt*, Berlin, 1875.

With the four cinchona derivatives, as with optochin, we have been able to show that migration of leucocytes follows almost immediately after the injection of a non-toxic dose of the drug into the peritoneal cavity of a mouse. The experiments reported in Table VIII were carried out by injecting 0.5 mg. of drug in a 1 cc. volume into the peritoneum of mice. At 1, 2, 3, 4, and 24 hours respectively, a small quantity of fluid was removed, diluted with a known amount of Turk's solution, shaken, and counted in the usual manner. Obviously, the drugs have a positive chemotactic influence on leucocytes in mice, since these migrate to the site of injection. Inasmuch as the cinchona compounds, as has been shown above, inhibit the phagocy-

TABLE VIII.

Chemotactic Influence of Aromatic Cinchona Derivatives.

Chemical.	Duration of observation.				
	1 hr.	2 hrs.	3 hrs.	4 hrs.	24 hrs.
(C 29) Hydroquinine chloroacetylanilide.	2,110*	2,340	5,740	4,000	9,400
(C 36) Dihydroquinine <i>p</i> -chloroacetylaminophenol hydrochloride.	3,520	5,360	5,140	4,300	No fluid.
(C 40) Dihydroquinine <i>m</i> -chloroacetylaminophenol hydrochloride.	2,720	3,020	4,620	5,000	" "
(C 110) Dihydroquinine 4-chloroacetylaminopyrocatechol hydrochloride.	3,860	4,200	6,800	9,840	63,680
(C 11) Optochin (ethylhydrocupreine).	740	1,660	22,300	5,360	No fluid.

* The numbers refer to cells per c.mm. of fluid.

toxis of staphylococcus and are also proven to have positive chemotactic characteristics, it would seem that the antiphagocytic action is due, to a large extent, to the paralysis of the function of the leucocytes and not to destruction of the cells.

DISCUSSION AND SUMMARY.

We are dealing, as the results show, with groups of chemicals, all of which, whether bacteriotropic or not, greatly inhibit the engulfing of *Staphylococcus aureus* by leucocytes. Not a sufficiently large number of experiments was performed in attempt to cure experimental staphylococcus infections to warrant any conclusion in regard to possible therapeutic activity against this organism. How-

ever, as will appear in another paper, the only group out of the seven which definitely possessed an *in vivo* bactericidal action against pneumococcus is that of the cinchona derivatives. Certain members of the other chemical groups studied, although bactericidal in a very high dilution,—chemicals in which the concentration of a non-lethal dose was many times greater than that required to kill multiple minimal lethal doses of organisms *in vitro*,—had no certain effect when bacteria and drug were injected simultaneously into the peritoneal cavity of a mouse. In fact, the treated mouse often died before the controls.

If we may assume,—leaving out of consideration the practical significance of *in vivo* chemical destruction and excretion following the injection of the drug into the animal,—that the failure of these chemicals to exhibit a benign influence on a systemic infection in cases in which the drug can be used in a bactericidal dilution, is due to their antiphagocytic property, only one step has been taken in analysis of the factors vital for the defense of the animal against a specific microorganism. Why do these chemicals inhibit leucocytic activity? Is it because of their influence upon complement, opsonin, or the leucocyte itself, or some special one function that determines the ability to ingest bacteria? Only further work can definitely settle this question and also determine whether or not such an analysis would be of practical importance in a rational development of chemotherapy.

The ideal chemotherapeutic agent may be one that has an *in vivo* bactericidal potency and a negligible or stimulatory phagocytic action in doses non-lethal for the experimental animal. However difficult such a drug may be to find, it seems unlikely that the ultimate success in chemotherapy will be so simple. Again, it is conceivable that a secondary action of a drug, although leucocytotropic and not bacteriotropic, may bring about conditions in the animal body that will enable it to throw off the invading organism. Or finally, a drug compatible with the forces necessary to the host's defense and possessing *in vivo* bactericidal action to a greater or less degree may be the chemical sought for, the goal toward which we should strive, to achieve a rational chemotherapy for infectious diseases.

CONCLUSIONS.

With certain members of the triphenylmethane dyes and leuco bases, safranines, phenazines, quinones, and cinchona groups of chemicals, there exists no consistent parallelism between the bacteriotropic activities and the organotropic and leucocytotropic activities.

All the chemicals tested possess a leucocytotropic action, as measured by the decreased ability of leucocytes to ingest staphylococci. This action against the functional activity of the leucocyte is more pronounced than the organotropism or bacteriotropism (for staphylococcus).

Four aromatic cinchona compounds, hydroquinine chloroacetylani-
lide hydrochloride (C 29), dihydroquinine *p*-chloroacetylaminophenol
hydrochloride (C 36), dihydroquinine *m*-chloroacetylaminophenol
hydrochloride (C 40), dihydroquinine 4-chloroacetylaminopyrocate-
chol hydrochloride (C 110), and optochin (C 11) are markedly anti-
phagocytic in their therapeutic dose. They possess a positive
chemotactic action for leucocytes when injected into the peritoneal
cavity of mice.

In the cases of *p*-methoxymalachite green (D 12), ethyl violet
chloride (D 32), and diaminoacridine sulfate (D 26) the condition
was approached in which the concentration of a non-lethal dose for
mice is staphylophagocytic and not leucocytotropic.

THE SIGNIFICANCE OF COLOSTRUM TO THE NEW-BORN CALF.

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The high mortality of calves during the 1st week of life has been the subject of researches for a number of decades. The chief symptoms preceding death are diarrhea, whence the name scours, inflammation of the umbilical stump (omphalitis), and multiple exudative arthritis. The concensus of opinion today is that there is a more or less close relation among these clinical manifestations and that, as pointed out by C. O. Jensen,¹ the chief infecting agents are races of *Bacillus coli*. In the latest summaries of handbooks covering this subject the emphasis is placed upon the infectious agents and all the measures that are recommended involve isolation, cleanliness, disinfection, and specific serum treatment.

Running parallel with these investigations but hardly influenced by them are studies on the transmission of immune bodies from mother to fetus and nursing. The fundamental studies of Ehrlich on ricin and abrin immunity transmitted to mice in the milk of immune mothers were followed by a series of investigations involving both normal and artificially induced antibodies in the mother's milk. These showed that not all species act alike, that some may transmit antibodies to the fetus *in utero*, others only in the milk.

The difficulty experienced in keeping calves alive which had not received colostrum from the mother led to a more thorough study of the effect of withholding this first milk. The significance of colostrum to the new-born calf is not set forth in medical or veterinary

¹ Jensen, C. O., in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, Jena, 2nd edition, 1913, vi, 121.

literature with any definiteness. This fluid is generally assumed by some as necessary to cause evacuation of the fetal fluids and solids from stomachs and intestines. Others think that a cathartic like castor oil may replace it. In formulating measures for preventing calf scours Hutyra and Marek,² probably basing themselves on the work of C. O. Jensen³ who stated in 1905 that calves die of enteritis if fed cooked or pasteurized milk during the first 24 hours of life, advise feeding the dam's colostrum for a day before heated milk is fed. They suggest that the fore milk be rejected since it might be infected. In the carrying out of the Bang system for the control of bovine tuberculosis, some have withheld colostrum, others have allowed the calf to suckle the dam immediately after birth, although the original method permits the calf to remain with the dam until it has taken the first milk.

Considerable work on the problem of feeding new-born calves has been done by W. L. Williams and associates.⁴ Although the factor of colostrum entered, it was complicated with the use of scours sera and the boiling or pasteurizing of milk. No definite experiments on the simple exclusion of colostrum from the diet and the association with these of adequate controls by which the unknown, uncontrolled factors might be balanced are reported.

J. Traum⁵ takes up the question of raising calves from a tuberculous herd by excluding the colostrum and milk of the reacting dam. Traum does not, however, state how many calves did not receive colostrum or milk immediately following the colostric period, for he writes that in the herd in which the experiment was going on "there were usually about 25 fresh cows yielding sufficient colostrum to frequently supply the calves born in reacting herd." Without doubt Traum did raise calves without colostrum, but he does not state the number as compared with those that did receive it nor give any subsequent history of the calves, nor does he report any losses by the way.

So far as it has been possible to examine the literature, the statement is warranted that no experiments dealing exclusively with the

² Hutyra, F., and Marek, J., *Spezielle Pathologie und Therapie der Haustiere*, Jena, 4th edition, 1920, i, 163.

³ Jensen, C. O., *Z. Tiermed.*, 1905, ix, 121.

⁴ Williams, W. L., et al., *Rep. New York State Vet. College*, 1914-20.

⁵ Traum, J., *J. Am. Vet. Med. Assn.*, 1921, lix, 755.

withholding of colostrum have been made which at the same time include a number of controls sufficient to eliminate highly virulent epizootics of scours. For this reason it has seemed justified to record the following experiments with a certain number of details concerning the individual calves gathered together in an appendix.

EXPERIMENTAL WORK.

In order to balance as far as possible the many unknown factors entering into the undertaking, two experiments were carried on simultaneously. One group of calves was to have colostrum, the other not. The calves were obtained from the same large herd. To make sure that none that had suckled the mother soon after birth should be included in the group not having taken colostrum, only those born during the day and definitely known to the attendant to be without food were chosen. These were removed with as little exposure as possible to the Department units, a distance of $1\frac{1}{2}$ miles. The calf was covered with a blanket, placed in a crate, and transported in a protected autotruck. The units were artificially heated. In the early experiments the calves were gently rubbed with cloths until nearly dry. Later they were washed with hot water containing some soda to soften the mucus adhering to the hair and thoroughly rubbed until dry. The umbilicus in later cases was snipped off if longer than 1 inch, gently manipulated to remove fluids, dipped in 0.1 per cent bichloride of mercury, and dusted with boric acid powder.⁶ In some the bichloride was omitted. The milk was fed soon after milking and was raw in all cases.

The group which took colostrum comprised ten calves. All of these survived the danger period and were kept various periods of time thereafter. Three died unexpectedly after 25, 38, and 45 days respectively (Nos. 757, 694, and 715). The protocols indicate that death was most probably due to some kind of poison and experiments are under way to clear up if possible the cause of death. Whatever this cause may have been, the evidence is quite conclusive that there was no ordinary infection involved, since in the two from whose organs cultures were made bacteria could not be demonstrated even though in one case (No. 715), death having occurred early Sunday

⁶ Williams, W. L., *Rep. New York State Vet. College*, 1913-14, 163.

morning, the autopsy was not made until after 28 hours refrigeration. A condition common to the three animals was the presence of punctiform hemorrhages throughout the intrathoracic portion of the thymus.

Of the second group of twelve calves which received no colostrum, nine died and three survived. These may be grouped according to the length of life. Seven of the calves died within 6 days. Thus four (Nos. 663, 685, 699, and 566) died within 3 days, one (No. 895) in $3\frac{1}{2}$ days, one (No. 894) in $4\frac{1}{2}$ days, and one (No. 665) in $5\frac{1}{2}$ days. One (No. 682) not included in the figures was too weak to take food and died within 2 days.

Before considering the gross appearances presented at autopsy, it should be stated that in new-born calves minute hemorrhages are almost regularly encountered in certain organs. They are rarely absent from the auriculoventricular valves of the heart. These have been made the subject of special study by Laura Florence.⁷ The mucosa of the leafy or expanded portion of the fourth stomach is regularly sprinkled over with petechiæ. The area involved varies from a few centimeters to the entire stomach. In some cases the hemorrhages are deep, in others superficial. Each spot is then capped with a bit of digested blood. Notably the pyloric valve is the seat of minute hemorrhages and in calves several months old shallow ulcers are occasionally still present on this valve. Less frequently the lowest 3 to 4 inches of the rectal mucosa is deeply congested and blood is now and then seen on the discharges. The ileocecal valve in rare instances is permeated with hemorrhages. In a few cases the mucosa of the rumen is involved in hemorrhages. If we except the heart valves, the hemorrhages, so far as studied, may be tentatively considered as mechanical in origin and due to conditions arising during parturition. Wherever in the present paper these hemorrhages are mentioned, the above facts should be borne in mind.

The appearances at autopsy differed somewhat from animal to animal and were due to differences in the blood content of the organs. The mucosa of the small intestine was in some instances pale, in others deeply congested in parts or throughout. The large intestine also varied in this respect. In all cases the small intestine was more deeply congested than the large. The other organs most involved were the kidneys. These varied from a nearly normal coloration and consistency to an intense congestion of the entire organ and a much firmer consistency. The urine taken from the bladder was free from blood or hemoglobin and contained only a trace of albumin

⁷ Florence, Laura, *Am. J. Dis. Child.*, 1922, xxiii, 132.

with one or two exceptions. The liver cells usually contained more or less visible fat. The spleen was normal in size and flabby. The thoracic organs presented nothing characteristic.

Coming to the bacteriology of these cases we meet a definite condition in all animals. The spleen, liver, and kidneys contained large numbers of *Bacillus coli*. Each loopful of tissue fluid contained hundreds of bacteria. There was undoubtedly to a certain extent

TABLE I.
The Results of Feeding and Withholding Colostrum.

No. of calf.	Colostrum fed +; not fed -.	Source of dam.	No. of pregnancy.	Source of milk fed.	Date of birth.	Died, killed, or sold.	Remarks.
663	-	Eastern.	1	601	Oct. 4	Died, Oct. 6.	<i>B. coli</i> septicemia.
664	+	Native.	1	600, 601	" 10	Sold, Nov. 2.	
665	-	Western.	1	601	" 11	Died, Oct. 16.	<i>B. coli</i> septicemia.
669	+	Native.	1	601	" 18	Killed, Feb. 13.	Normal.
680	+	"	1	678	Nov. 1	Sold, Dec. 10.	
681	-	Eastern.	3 (?)	?	" 2	Killed, Nov. 5.	<i>B. coli</i> septicemia.
685	-	Native.	3	?	" 9	Died, " 11.	" " "
687	+	Western.	1	679	" 9	Sold, Dec. 10.	
694	+	Eastern.	1	679	" 25	Died, Jan. 2.	No cultures. Poison (?).
697	-	"	3	679	" 29	Sold, Feb. 2.	
698	+	Native.	4	678	Dec. 6	" " 2.	
699	-	"	3	678	" 6	Died, Dec. 8.	<i>B. coli</i> septicemia.
715	+	"	1	712	Jan. 5	" Feb. 19.	Cultures sterile. Poison (?).
717	-	Western.	1	678	" 11	" Jan. 22.	<i>B. coli</i> septicemia.
716	-	Native.	3	712	" 11	Killed, Mar. 16.	Normal. Cultures sterile.
718	+	Western.	1	678	" 12	Sold, Feb. 2.	
757	+	Native.	3	678	" 23	Died, " 17.	Cultures sterile. Poison (?).
759	-	"	2	678	" 25	Killed, " 21.	Miscellaneous bacteri- emia.
893	+	"	1	712	Feb. 9	" Mar. 28.	Normal.
894	-	"	1	712	" 9	Died, " 13.	<i>B. coli</i> septicemia.
895	-	Eastern.	4	678	" 10	" " 13.	" " "
566	-	Native.	1	678	" 10	" " 12.	" " "

multiplication after death but, as will be seen farther on, these bacilli are present before natural death in abundance. Other organs were not cultured except in several cases certain joints which also yielded *Bacillus coli*.

The calf (No. 682) which died within 2 days and whose stomachs contained no food presented the same *Bacillus coli* septicemia.

No. 681 is of interest. On the 4th day of life it was too weak to get up and take its food. It would have presumably died during the night and it was therefore killed. The kidneys were firmer than normal and deeply congested. Cultures showed the presence of large numbers of *Bacillus coli* in the spleen, liver, and kidneys.

One calf (No. 717) died when 11 days old of a *Bacillus coli* septicemia. Cultures from one tarsal joint contained only *Bacillus coli*.

One calf (No. 759) which survived became lame. Several joints were involved. It was killed when 27 days old. There was a purulent fluid and a fibrin mold in one joint. The kidneys presented numerous whitish sclerotic foci. Cultures from the spleen remained sterile. Those from the kidneys and liver represented mixed infections, those from the joint exudate pure *Bacillus coli*. Of the two remaining calves, one was sold to the butcher in fine condition when 2 months and 3 days old. The other was killed when 2 months and 5 days old. The organs presented no abnormalities.

In Table I the cases are arranged according to the dates of birth. It shows that the calves deprived of colostrum and the controls were well balanced as to season.

DISCUSSION.

The data presented in the foregoing cases lead to certain definite inferences. The calf deprived of colostrum lacks something which permits intestinal bacteria to invade the body and multiply in the various organs. The rapidity and duration of this multiplication determine the fate of the calf. In most cases a rapidly fatal septicemia is the result. When the resistance is greater life may be prolonged or the animal survive indefinitely. The surviving animal may completely subdue the invading bacteria and develop into a normal calf or else localizations arise which involve, in the cases

here presented, the joints or the kidneys or both organs as in No. 759. The joint lesions are due to a fibrinous and purulent exudation. The bacteria present belong to *Bacillus coli*. The end-result of the localization in the kidneys is a fibrosis limited to the cortex with localized destruction of the secreting tissue. This occurs in foci of varying extent. The largest seen were about 1 cm. in diameter. This pathological condition, which was observed in 1917 associated with pneumonia due to *Bacillus actinoides*,⁸ may now be considered as partly cleared up and it remains to study the early stages leading to fibrosis. The precise etiological factor may be *Bacillus coli* or some organism associated with it.

The bacteria isolated from the various cases and which were present in very large numbers in those that died and in fairly large numbers in those that were killed and cultured within 2 hours belong to that variety of *Bacillus coli* which is non-motile, indole-producing, and which fails to act on saccharose. This particular type of *Bacillus coli* has been under observation since 1917 and has been frequently isolated from the small intestines of calves dying in the first few days of life.

The experiments described are in a sense fundamental and many subsidiary questions are left unanswered. One of these is the effect of the ordinary milk of the different cows fed, whether protective and if so to what degree. In the table, the cow's milk fed is indicated by a number and it will be seen by consulting this that the source of the milk is not a determining factor in the experiment. Another question to be answered is whether it might not be possible by using extreme and unremitting care to raise most calves deprived of colostrum and how many of these would develop scours, joint lesions, omphalitis, or pneumonia in due time.

In general it may be concluded that the function of the colostrum is essentially protective against miscellaneous bacteria which are harmless later on when the protective functions of the calf have begun to operate and accumulate energy. There appears to be no function inherent in colostrum which controls development or growth or which is essential to the starting of the mechanism of digestion,

⁸ Smith, T., *J. Exp. Med.*, 1921, xxxiii, 441.

since calves not having had colostrum appear to do as well as the others when the infection has been overcome.

The results obtained clear up much of the mystery surrounding the mortality and morbidity of very young calves. They explain why disease may appear when there is no evidence of introduction from without, and why the causes of disease of young calves up to 2 months old have been referred to the first days of life and to prenatal infection, especially where infectious abortion is prevalent. They moreover point out why so many different kinds of bacteria have been regarded as responsible for calf scours. Obviously the most invasive of the flora of any herd will dominate the bacteriology and different organisms will predominate in different territories unless the interchange of animals is very active, in which case the same flora will probably be found in many herds. The results also explain mixed infections, since the portal of entry from the intestinal tract is open.

These results furthermore show the inadequacy of all attempts at prevention based on keeping the invading bacteria away. These live in the normal cow, possibly some in the udder itself, and must be considered ubiquitous. They also demonstrate the futility of administering specific serum unless the dominating pathogenic organisms of the herd are known and used in its preparation.

It is not claimed as a result of these experiments that all colostrum-fed calves will resist the various forms of disease described, for we know that they prevail in spite of normal feeding at birth. There may be degrees of virulence in the intestinal, respiratory, and udder flora of cows against which normal feeding is quantitatively inadequate. It so happened that in the experiments reported no highly virulent types were present and therefore the dividing line between the calves protected by colostrum and those not so protected was unusually well defined.

CONCLUSIONS.

All of ten calves which were permitted to take colostrum after birth survived. Eight out of twelve calves which did not get colostrum died and one was killed moribund. One calf, killed on the 27th day, harbored miscellaneous bacteria in its organs. The kidneys

were sclerotic and one joint diseased. Of the remaining two calves, one had transitory joint troubles, the other rhinitis. One was sold and the other killed when 2 months old. In the latter the organs were normal and sterile.

Notes on the Individual Calves.

Calf 566.—Male, born Feb. 10, 1922, at 2.30 p.m., of a native cow in her second pregnancy. Calf removed from the dam immediately after birth and taken to the Department buildings at 4 p.m. There it was dried off by gentle friction with cloths. The umbilical cord was treated with bichloride of mercury and powdered boric acid. Fed $1\frac{1}{2}$ lbs. milk (of Cow 678) from a bottle in evening.

Feb. 11. Calf fed three times today, drinking in all $4\frac{1}{2}$ lbs. of milk. Temperature 39.0–39.5°C.

Feb. 12. Temperature 39.2°C. early. Calf drank first meal. Died about 11 a.m. Refrigerated at once until Feb. 13. The autopsy showed little of significance except the following.

The fourth stomach contained a milk curd the size of a small apple, some smaller clots, and milky fluid. Punctiform hemorrhages in leafy portion. Rectum deeply congested. Liver cells contain much fat. Extensive subendocardial extravasations of blood. Urine taken from bladder contains a trace of albumin. Both metatarsal joints contain some light yellowish fluid and soft mucoid flakes consisting largely of polynuclear leucocytes.

Cultures from spleen, liver, and kidneys are overgrown with a heavy glistening layer which in subsequent dilution cultures proves to be the saccharose-non-fermenting *B. coli*. One of several tubes inoculated with the exudate from the tarsal joint contains numerous colonies of the same organism.

Calf 663.—Born Oct. 4, 1921, at 11.50 a.m., of an eastern cow. First calf since introduction into herd. Calf not allowed to suckle the dam, and removed to Department unit at 1.35 p.m. Fed $2\frac{1}{2}$ lbs. of milk from Cow 601 at 5.30 p.m. Abundant discharge of meconium during the night. Fed next morning. The discharges became softer and were yellowish in color.

Oct. 6. The calf was weak, unable to get up, and still scouring. Respirations shallow, about 60 per minute. Opaque, whitish, mucoid discharge from left nostril. Calf died at 11 a.m.

Autopsy.—Weight 67 lbs. Umbilical cord shrunken to a dried black tape-like body. Hemorrhagic discoloration of sheath and suspensory ligaments of umbilical artery stumps. Umbilical vein patent within abdomen to liver. Contents a normal blood clot. Beneath epicardium around base of heart, some hemorrhages. Hemorrhages within heart valves (auriculoventricular).

Digestive tract: Rumen normal. Fourth stomach contains white curds and some opalescent fluid. Leafy portion sprinkled with faded petechiæ. Pyloric portion and valve deeply congested. Uniform deep congestion of entire length

of small intestine. Bits of mucosa examined microscopically show complete injection of the network of capillaries in the villi. Large intestine congested but less so than the small. Liver shows considerable intracellular fat. The spleen is flabby, somewhat congested. Kidneys deeply congested. Urine taken from bladder is faintly clouded, yellow, slightly acid. Contains a trace of albumin and no sugar. Specific gravity 1.026.

Cultures from spleen and liver indicate large numbers of *B. coli* evidently pure. From the contents of a loop of the small intestine dilutions spread on agar show the same type of colonies. Those from a liver culture replated found to be colon bacilli non-motile, not fermenting saccharose.

Calf 664.—Born Oct. 10, 1921, at 5.20 p.m., first calf of a native cow, A 945. The dam had been vaccinated with a living culture of *B. abortus* before being bred. The parturition was normal and a guinea pig inoculated with material from a uterine swab was negative for *B. abortus*.

The calf remained with dam until 8.20 a.m. next day when it was removed to an isolation unit at the Department and fed milk from Cows 601 and 600. The temperature taken twice daily fluctuated between 38° and 39°C. The calf remained well and was sold Nov. 2.

Calf 665.—Born Oct. 11, 1921, at 2.50 p.m., of a midwestern cow introduced into the herd Sept. 27. Calf removed immediately after birth to a Department unit at 3 p.m. and fed milk from Cow 601.

Oct. 12. Calf drank 5 lbs. of milk in morning and 4 lbs. in evening. The temperature was 39° and 39.3°C.

Oct. 13. Calf drank 4 lbs. of milk morning and evening. Temperature 39° and 39.4°C.

Oct. 14. Calf drank 4 lbs. in morning. Feces soft, yellow, streaked with blood. Evening food withheld.

Oct. 15. Given 4 lbs. of milk. Temperature 39.8°C. Calf had a chill. Respirations 48; pulse 100. Feces soft with fetid odor. Discharge from both nostrils, mucous in character. Temperature, 5 p.m., 40.2°C. Calf drank 2 lbs. of milk. Temperature 39.6° at 9 p.m. Calf can stand up but unable to move legs freely.

Oct. 16. Temperature 38.6–39.1°C. during the day. Calf takes 1 lb. of milk, is very weak, unable to hold up head. Feces soft, streaked with blood. Legs cold. Dies at 11 p.m. and is placed in refrigerator.

The autopsy made early next morning. Weight of calf 88 lbs. The only noteworthy feature is an intense congestion of the medullary portion of kidneys. Urine from the bladder has a specific gravity of 1.011, yellow, cloudy, alkaline, albumin 0.2 per cent Esbach; sugar absent.

Cultures from spleen, liver, and kidneys show large numbers of colonies of non-motile bacilli like those from Calf 663. They ferment dextrose and lactose but not saccharose.

Calf 669.—Male, born Oct. 18, 1921, 1.20 p.m., first calf of a native cow. Remained with dam until 5 p.m. Then taken to an isolation unit at the Depart-

ment. The dam had been vaccinated same as dam of Calf 664. Calf was not fed in the evening. It refused milk next morning. In the evening it drank 2 lbs. The milk came from Cow 601.

The calf continued normal with temperature fluctuating between 37.5° and 38.5°C. until Nov. 5, when the daily taking was discontinued.

The calf was killed Feb. 14, 1922. It weighed 225 lbs. No lesions were found.

Calf 680.—Male, born during the night of Nov. 1 to 2, 1921, first-born of a native cow. The calf, which had suckled, was removed in the morning to an isolation unit at the Department and fed milk of Cow 678.

From Nov. 2 to 4, the stools were very liquid. Some diarrhea was again noticed on Nov. 21 and 22. The temperature fluctuated between 38° and 39°C. at this time. Otherwise the calf remained well and thrifty until sold Dec. 10.

Calf 681.—Male, born Nov. 2, 1921, 2 p.m., of a native cow. Probably the third or fourth calf. Calf kept from suckling the dam and brought over to a Department unit at 4.30 p.m. Fed 2 lbs. of milk.

Nov. 3. Fed 3 lbs. of milk in the morning. Large mass of feces passed with tendency to become liquid. Discharge from nose. Temperature 38.7–39.0°C.

Nov. 4. Takes 3 lbs. of milk. Discharge from nostrils more profuse. Calf grows weaker and unable to stand later in day. Temperature 39.2–39.7°C.

Nov. 5. Calf very weak, refuses food. Is unable to get up. In this case it was thought best to kill the calf to eliminate postmortem changes. Killed by stunning and cutting vessels of neck. Weight 73 lbs. The autopsy was negative except for the following.

The fourth stomach contained a mass of coagulated milk about 8 cm. in diameter. The mucosa of this organ and of the small and large intestines normal. The liver cells contain considerable fat. The kidneys are slightly enlarged, very firm to the touch, and deeply congested throughout. Urine from the bladder is yellowish, somewhat cloudy, slightly acid, specific gravity 1.030, albumin 0.15 per cent. Sections of the kidney show a complete injection of the entire capillary system. Those of spleen show numerous hemorrhages in the parenchyma.

Cultures were made from spleen, liver, and kidneys as follows: A bit of tissue about the size of a pea or small bean was transferred to an agar slant, rubbed over the surface, and deposited in the condensation water. A platinum loop dipped into this condensation water was rubbed over the slanted surface of a second tube. The numbers of colonies in the first tubes were countless. Those from the second tubes were 3 for the spleen, 15 for the liver, and 6 or 7 for the kidney. Dilutions from an intestinal loop planted in agar yielded the same kind of colonies; namely, the saccharose-non-fermenting *B. coli*.

Calf 682.—Born Nov. 2, 1921, before 6 a.m., of an eastern cow in her third pregnancy. This calf weak at birth remained so and was unable to take food. It grew weaker and was killed when about 33 hours old, Nov. 3, 2.30 p.m., to determine the presence or absence of *B. coli* septicemia.

The autopsy showed the absence of any food in the stomachs. These still contained the fetal fluids, viscid, bile-stained. The mucosa of nearly the entire

fourth stomach and pyloric valve was sprinkled over with minute, irregularly star-shaped hemorrhages, about 1 mm. apart. No surface hemorrhage. The small intestine nearly empty, normal. The lowest 24 inches of large intestine sprinkled with punctiform hemorrhages merging into a deep universal congestion in the rectum. The liver cells contained much fat. Other organs appear normal.

The cultures from spleen, liver, and kidney showed coalescing layers of growth in those in which bits of tissue had been placed. Those inoculated with a loop of condensation water from the preceding contained 6 or 7 colonies (spleen), 1 colony (kidney), and 6 or 7 colonies (liver). Dilutions of contents of an intestinal loop spread over agar showed the same type of colony. The bacilli were of the saccharose-non-fermenting type.

Calf 685.—Male, born Nov. 9, 1921, at 2 p.m., of a native cow in her third pregnancy. Calf removed from dam immediately after birth. At 3.10 p.m. placed with a cow which had calved 5 days ago. Calf began to suckle at 3.20 p.m. At 4 p.m. it was taken to a Department unit.

Nov. 10. Calf took 3 lbs. of milk early. Passed dark greenish masses of meconium. Feces tending to become liquid during the day. Temperature rose from 38.5°C. in morning to 39.6°C. in the late afternoon. Calf refused to take but a little milk late in the day, now very weak.

Nov. 11. Calf died at 5 a.m. From the autopsy record the following may be mentioned.

The umbilical vessels show only the usual amount of hemorrhagic discoloration. Rumen empty, normal. Fourth stomach normally distended with an opalescent, watery fluid and many small casein clumps. An area about 10 cm. in diameter of the leafy portion sprinkled with surface hemorrhages. Faded deeper hemorrhages in rest of leafy portion. In the small intestine there are well defined areas of deep congestion through the entire tube. There are linear congestions on crests of longitudinal folds of large intestine. Liver cells contain considerable fat. Spleen flabby. Some papillæ of kidneys congested. Heart and lungs negative.

Cultures from spleen, liver, and kidney show the same invasion of *B. coli* as in the preceding cases.

Calf 687.—Male, born Nov. 9, 1921, at 4.15 p.m. Remained with the dam until Nov. 10, 11.10 a.m., when it was removed to the Department animal house. The dam had been brought from Michigan in October. The calf refused to take milk (from Cow 679) the first evening, but drank next morning and thereafter. The temperature fluctuated between 38° and 39°C., rarely falling as low as 37.5°C. Nothing abnormal was observed. On Dec. 10 the calf was sold to the butcher.

Calf 694.—Born Nov. 25, 1921, at 1.30 p.m. Dam an eastern cow. Not known how many calves preceded this one. There was some difficulty in getting the calf to suckle the dam. At 4 p.m. it was induced to take colostrum from two quarters for about 10 minutes. At 4.30 p.m. it was transferred to the Department units. The subsequent feeding occasioned no special difficulties.

Up to Jan. 2, 1922, the temperature maximum and minimum were 39.8° and 37.5°C. Most of the morning and afternoon readings fluctuated between 38° and 39°C.

The calf was found dead unexpectedly at 7 a.m., Jan. 2. It had not been seen since 11 p.m., Jan. 1. It was at once placed in the refrigerator until Jan. 3. The autopsy presented the following points.

The mucosa of the digestive tract was abnormally pale. The fourth stomach contained a firm milk coagulum in which were enmeshed pieces of twine eaten by the calf from the straw bedding. There was more than the usual bile staining in the small and large intestines. The heart cavities contained soft dark clots and the endocardium was deeply suffused with blood coloring matter. The spleen was very flabby and of normal size. Some large bacilli (postmortem?) present. The liver was yellowish in color. There were a moderate amount of fat in the liver cells and some orange pigment. Bacteria not seen in films. The kidneys on section showed closely set, pale, linear streaks, passing vertically through cortex. In fresh scrapings the nuclei of the convoluted tubule epithelium contained each an irregular, refringent block or concretion. The thymus within the thorax was densely studded with hemorrhages up to 2 mm. in diameter. The cervical portion was free from them. Cultures were not made.

Calf 697.—Female, born Nov. 29, 1921, 2 p.m., of a native cow in her third pregnancy. The calf was unable to stand up for a time. At 2.45 p.m. it was transferred to the Department units and gently dried by rubbing with cloths. At 4 p.m. it was allowed to suckle another cow (No. 600) but fed poorly and obtained little.

Nov. 30. Temperature 38.5° and 39°C. Took 2½ lbs. of milk morning and evening. Voids large masses of meconium. The milk came from Cow 679. Dec. 1. Temperature 39° and 39.7°C. Drank 3 lbs. milk morning and evening. Mucopurulent discharge from nostrils. Dec. 2. Temperature 39.3° and 40.2°C. Food as yesterday. Dec. 3. Temperature 40°C. Discharge from nostrils abundant, thick. Feces normal. Dec. 4. Temperature 39.3° and 39°C. Food as before. The calf soon recovered from the rhinitis and gained weight normally. It was used in another experiment and sold to the butcher in fine condition Feb. 2, 1922. The inspector reported organs normal.

Calf 698.—Male, born Dec. 6, 1921, between 12 and 1, noon, fourth calf of a native cow. The calf was allowed to take the colostrum and then removed at 2.20 p.m. to the Department buildings.

The calf refused to drink next day until evening when some milk was slowly poured into its mouth. At 11 p.m. it drank about 12 ounces from a bottle. On the following day it began to take food in larger quantities. The temperature rose to 39.6°C. on Dec. 7. Thereafter it fluctuated between 38° and 39.5°C., falling to 37.4°C., Dec. 15. Temperature taking discontinued after Dec. 23. Calf sold to butcher Feb. 2, 1922. Inspector reported the organs normal.

Calf 699.—Male, born Dec. 6, 1921, at noon, of a native cow in her third pregnancy. Calf removed at once to Department buildings and dried off by rubbing with cloths. Fed about 2 lbs. of milk from Cow 678.

Dec. 7. Calf drank about 2½ lbs. of milk morning and evening. A large amount of feces was passed during the day, gradually assuming a liquid condition. Temperature 38.2° and 39.7°C. Dec. 8. Calf died at 8 a.m. At the autopsy the following appearances were noted.

The mucosa of pharynx and larynx cyanotic. A milk curd about 3 to 3½ inches in diameter in the fourth stomach and some milky fluid. Mucosa dark bluish red. The mucosa of small intestine deeply injected throughout. Microscopic examination shows complete injection of capillary system of villi. The large intestine similarly congested with intensification in the rectum. Bowels practically empty. Thoracic organs show nothing noteworthy. Liver cells contain considerable fat. The spleen is splashed with subcapsular hemorrhages. The kidneys are deeply congested throughout. Urine taken from the bladder is clear, yellow, acid. Specific gravity 1.025. A trace of albumin present.

Cultures from spleen, kidney, and liver show as heretofore the presence of large numbers of colon bacilli, not fermenting saccharose. The same type isolated from two segments of the small intestine. Other species not in evidence.

Calf 715.—Female, born Jan. 5, 1922, about 4 a.m., of a native cow in her first pregnancy. The calf was left with dam until 4 p.m. and then brought to the Department units.

The calf was fed milk from Cow 712 the next day and feeding continued three times daily. The temperature remained normal and the appetite good. From Jan. 18 on it was taking 12 lbs. of milk. Jan. 26. *B. bovis septicus* was introduced into one nostril and the reaction following was slight. It was found dead unexpectedly Feb. 19 at 5.30 a.m. when 45 days old. The watchman reported behavior of the calf similar to that of No. 757 before death. The calf was placed in the refrigerator until Feb. 20.

The only noteworthy changes observed at the autopsy were as follows: The fourth stomach contains milk clots mixed with twine picked out of bedding. The mucosa is partly digested. Extensive subendocardial hemorrhages in the left ventricle. All cavities contain soft dark clots. The spleen is very flabby and not enlarged. The liver cells contain large fat globules. The kidney cortex is regularly marked with grayish streaks. The intrathoracic portion of the thymus is permeated with hemorrhages 1 to 2 mm. in diameter.

Cultures of bits of tissue from liver, spleen, and kidneys in plain and blood agar slants and in fermentation tubes remain indefinitely sterile. Similarly, cultures from the heart's blood.

Calf 716.—Female, born Jan. 11, 1922, 1.15 p.m., of a native cow in her third pregnancy. Calf taken from cow and removed to the Department buildings, rubbed until nearly dry. Fed 1½ lbs. of milk from Cow 712 in evening from a nursing bottle.

Jan. 12 to 16. The calf takes milk regularly and is fed three times a day. The temperature is within the normal range. Jan. 17 to 18. Temperature 39.5°C. Evidence of joint involvement in left elbow and tarsal joints. Calf constipated. Jan. 19. Calf moves more easily about stall. Is taking 9 lbs. of milk daily in three doses. Feb. 1. Slight lameness in left fore leg and some weakness in hind limbs. Feb. 11. Swelling of left tarsal joint probably a distension of synovial sheaths. Feb. 28. Temperature has been normal to date. The joint troubles have practically disappeared. This calf was killed Mar. 16, when 2 months and 5 days old. There were no abnormal appearances of the organs and the cultures made as heretofore remained sterile.

Calf 717.—Male, born Jan. 11, 1922, at 4 p.m., of a midwestern cow. First calf since coming into this herd. The calf was removed at 5 p.m. to the Department buildings and dried off by gentle friction with cloths. Fed at 5.30 p.m. from a nursing bottle about 1 lb. of milk from Cow 678.

Jan. 12 to 19. Fed three times daily about 2 lbs. of milk each time. Jan. 20. Temperature up to 40.2°C. this morning. Calf is very weak but is taking its food as usual. Constipated. Temperature 41°C. at 8 p.m. Respirations short and irregular. Jan. 21. Calf took its food three times today. Is slightly better. Umbilicus slightly swollen and tender. Jan. 22. Calf too weak to get up and take its food. Dies at 10 a.m. Refrigerated at once until next day.

Jan. 23. Autopsy. Weight of calf 92 lbs. Umbilicus has a dry black tape still attached. Base slightly thickened and indurated. When tape removed, a circular area is exposed covered with a thin layer of pus and communicating with a closed diverticulum with purulent contents. No communication with umbilical vein, which is closed. The digestive tract normal with exception of some deeply congested patches of mucosa in ileum. Lungs slightly edematous and congested. Some small fibrin masses in pericardial sac and a few hemorrhages under epicardium. Liver, spleen, and kidneys softer than normal. Slight vascular injection of tissues within tarsal joints. Urine from the bladder contains only a trace of albumin. Specific gravity 1.012.

Cultures from spleen, liver, and kidneys show presence of large numbers of *B. coli*. Many colonies of the same bacilli in pure culture obtained from the fluid in one tarsal joint.

Sections of fixed and hardened tissue presented the following data. Spleen: Foci or colonies of *B. coli*-like rods scattered regularly through the section. Liver: One minute abscess in the section. Every liver cell contains one or more (fat) vacuoles. Each vacuole contains a central coccus-like body stained reddish (fat crystals). Kidney: Fibrin thrombi in small vessels of cortex. Occasional foci of polynuclear cells, interstitial hemorrhages, and *B. coli*-like rods filling capillaries.

Calf 718.—Male, born Jan. 12, 1922, at 7.45 a.m. Dam brought from Michigan Nov. 20, 1921. To make certain that the calf had received colostrum it was placed with the dam again at 11 a.m. and allowed to suckle the four quarters, in

all for 20 minutes. It was then brought to the Department buildings. The milk fed was from Cow 678. No difficulty was experienced in starting the artificial feeding. The maximum and minimum temperatures until Feb. 2 were 38.8° and 37.8°C. On this day it was sold to the butcher. Organs were reported normal by inspector.

Calf 757.—Male, born Jan. 23, 1922, at 9 a.m., of a native cow in her third pregnancy. The calf suckled the dam about 15 minutes before removal to the Department units. The cord was treated in the same way as that of Calf 893. The calf was fed three times daily with milk of Cow 678. The temperature and appetite continued normal. On Feb. 13, being in fine condition, it was used in another experiment, *B. bovis septicus* was introduced into a nostril. The reaction was slight.

On Feb. 17, when the calf was 22 days old and had been without abnormal symptoms thus far, it suddenly (1.55 p.m.) became very excited. It moved in a circle from left to right and lowed continually. The respirations were noisy, labored, the tongue protruded slightly, and there was more or less frothy mucus about the muzzle. The calf was forcibly restrained by being thrown and held. After a time the convulsive movements became less pronounced and the dyspnea more marked. It soon was unable to rise after being released and died 15 minutes after it had been discovered in this condition. This calf up to the time of the seizure had been in excellent condition and had even taken its milk early on the day of death as usual.

The autopsy showed nothing abnormal, except numerous small hemorrhages through the intrathoracic portion of the thymus and some minute hemorrhages under epicardium. The fourth stomach contained pieces of twine in the milk coagulum.

Cultures on plain and blood agar slants with bits of tissue from spleen, liver, and kidneys and with heart's blood, sealed, remained free from growth after 5 days incubation.

Calf 759.—Male, born of a native cow in her second pregnancy, Jan. 25, 1922, 3.20 p.m. Removed from the dam before suckling and taken to the Department buildings where it was dried off by gentle rubbing with cloths. The umbilical cord had been torn off close to the skin. The exposed area was washed with 0.1 per cent mercuric bichloride and dusted with powdered boric acid. The calf was very weak and scarcely able to stand up. Fed from bottle $\frac{3}{4}$ lb. of milk from Cow 678. Fed again at 10.35 p.m.

Jan. 26. Fed three times today. Dry meconium being passed. Calf stronger. Jan. 27. Temperature 39°C. Takes food regularly. Soft yellow feces passed in large amounts, becoming liquid. Jan. 28. Temperature 39.1–39.2°C. Feces liquid. Jan. 29. Temperature 39–38.4°C. Lameness appears in right carpal and left tarsal joints. Calf quite weak. Feb. 4. Temperature has ranged close to 39°C. Some swelling of right carpal joint and stiffness in hind limbs.

Feb. 21. Calf has been improving and taking about 12 lbs. of milk daily. Killed today by stunning and cutting vessels of neck. Weight 87 lbs.

The autopsy showed the following abnormalities. In the right cephalic lobe of lungs some collapsed areas, each 4 to 5 mm. in diameter. Both kidneys spotted with white patches varying from mere specks to areas of a square centimeter. These areas correspond to the bases of solid foci reaching to medulla. A vertical section through these foci is either linear, square, fan-shaped, or oval. The involved tissue is white, smooth, glistening on section and quite firm. The distribution is irregular, not associated with any structures in the cortex. The urine taken from bladder is clear amber-colored, alkaline, specific gravity 1.020. A trace of albumin is present. The liver shows a distinct fatty zone on the periphery of the lobules.

In the right radiocarpal articulation a small quantity of an opaque fluid containing soft mucoid flakes together with a firm whitish fibrinous mold of a portion of the joint cavity, loose, and easily removed with forceps. The exudation and edema extend from the joint up the external aspect of the leg (radius) between the muscles for 3 to 4 inches. The joint exudate consists of polynuclear leucocytes.

Cultures were made with bits of tissue from spleen, liver, kidney, and the diseased joint. The spleen culture remained sterile. In the liver and kidney tubes several species of bacteria appeared, indicating a miscellaneous infection of the body. The joint cultures contained *B. coli*.

Calf 893.—Male, born Feb. 9, 1922, about 6 a.m. First-born of a native cow. Calf at first removed from dam as not having suckled, but owing to uncertainty in this respect, it was allowed to suckle dam at 11 a.m. for 20 minutes and then taken to the Department buildings. The umbilical cord was snipped off, leaving about 1 inch. This was dipped in 0.1 per cent mercuric chloride and covered with powdered boric acid. First meal taken the same evening was milk from Cow 712. There was no difficulty in feeding. The temperature was taken twice daily until March when the calf was used in another experiment. During this time, the maximum and the minimum temperatures were 39.4° and 37.7°C. The calf was killed Mar. 28. The only abnormality found was pigmentation of the mucosa of cecum, along the course of the larger vessels.

Calf 894.—Male, born Feb. 9, 1922, 6.30 a.m., of a native cow in her first pregnancy. The calf was removed from the dam but was not taken to the Department buildings until about noon. It was probably exposed and chilled in this interval. It was then gently rubbed with cloths and the umbilical cord treated as in earlier cases. Fed at noon and night from a bottle with milk from Cow 712. Feb. 10. Fed three times today. Temperature 38.8° and 39.2°C. Large amounts of dark greenish feces passed today. Feb. 11 to 12. Temperature range 38.8–39.4°. Tendency towards diarrhea. Feb. 13. Calf is very weak. Takes milk twice, but unable towards night to stand. Feces fluid. Dies at 10.30 p.m. Placed in refrigerator until next morning. Weight 73 lbs.

Autopsy negative except for petechiæ in fourth stomach and a deep congestion of the mucosa of ileum. Tubes inoculated from spleen, liver, and kidneys show large numbers of colonies of *B. coli*.

Calf 895.—Male, born about 7 a.m., Feb. 10, 1922, of an eastern cow in her fourth pregnancy. Removed promptly from the dam and taken to the Department units at 10.20 a.m. where it was dried and umbilicus treated. The calf was fed at noon and evening with milk from Cow 678.

Feb. 11. Large amounts of relatively firm meconium discharged last night. Fed three times today. Temperature 38.8–39.7°C. Feb. 12. Fed as before. Calf growing weak and unable to stand later in day. Temperature 39.1–39.5°C. Feb. 13. Calf unable to stand up and drink. Nostrils filled with mucus. Temperature falling, 38.4–37.1°C. Died at 11 p.m. and refrigerated soon after.

Feb. 14. Autopsy presents the following points. The umbilical vein just beneath abdominal wall is distended with a normal clot about 2 cm. in diameter. No communication with exterior. Fourth stomach contains some curds and milky fluid. Many superficial hemorrhagic points in leafy portion. Rectum deeply congested. Some dried yellowish fecal matter in it, blood-stained. Both carpal joints contain several cubic centimeters of a turbid fluid with flakes made up of polynuclear leucocytes.

Interarticular surfaces of tibiotarsal joints deeply congested. Cultures from spleen, liver, and kidneys show coalescent layers of growth. Cultures from metatarsal joints, large numbers of isolated colonies. These and the growth from spleen were replated and found to be the same type of *B. coli*.

INFLUENCE OF GLANDS WITH INTERNAL SECRETION ON THE RESPIRATORY EXCHANGE.

IV. EFFECT OF SUPRARENAL INSUFFICIENCY IN CATS.*†

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Marine and Baumann in 1921 demonstrated a significant and sustained increase in heat production in the majority of rabbits following suprarenalectomy or freezing the suprarenals (1). The importance of this type of reaction in general physiology and special pathology makes it desirable to ascertain whether this relationship between the suprarenal gland and basal metabolism is peculiar to some feature of organization in this species. The literature contains no satisfactory evidence on this point. Golyakowski, working with dogs and utilizing an undescribed method of ligation, studied the respiratory exchange (2). He reported an increase in CO_2 and heat production in eight of twelve dogs. However, the importance to be attached to this observation is greatly diminished by the fact that the oxygen intake was not altered. On the other hand, Aub reported a decrease in heat production in three cats following suprarenalectomy but he did not investigate the effect of sublethal crippling of the suprarenals (3). No other references bearing in any way on this subject could be found.

The rabbit has relatively great normal temperature variations and it usually has accessory suprarenal masses, differing from the higher mammals in both points. The cat is a typical member of the latter group. The temperature is fairly constant and accessory

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suprarenals are rare. Therefore, total removal is followed by death usually within 2 to 5 days (4). Also in its diet and its fat metabolism the cat differs from the rabbit and resembles the organization of man. This point takes on special interest in view of the recent demonstration by Marine and Baumann that the thyroid function is essential for the increase in heat production during suprarenal insufficiency (5), taken in conjunction with the connection between the thyroid and the metabolism of fat pointed out by McCarrison (6). Therefore, in this report we wish to record the changes in heat production observed in cats following varying degrees of suprarenal injury produced by partial extirpation, vessel ligation, freezing, and various combinations of these procedures.

Methods.

Cats were kept in the laboratory under uniform conditions of régime and diet for at least 3 weeks before determination of the respiratory exchange and they were run in the respiration chambers several times for training during this period. The gaseous metabolism was measured with the Haldane apparatus as modified by Marine and Lenhart (7). The room temperatures were kept relatively constant. The maximum variation (with the exception of October 5) was 19–24°C. with the mean of 21°C. Determinations were always made at least 15 hours after the last feeding; and a sufficient number of them over a period of at least 2 weeks was obtained to determine the normal range for the individual cat under the given conditions as regards season, food, state of nutrition, etc. About 10 per cent of the cats were rejected for this work because the control rates were too irregular. Those used were very quiet after becoming accustomed to the procedure, and frequently slept throughout the entire period in the metabolism chambers which was always 2 hours. Any periods in which the animal was restless were discarded.

Three methods were used in our attempts to cripple the suprarenal function:¹ (a) partial excision; (b) ligation of blood vessels; and (c) freezing.

Partial Excision.—This was the method first attempted as it would appear *a priori* to be the most exact means of producing varying

¹ All operations were performed under ether anesthesia.

degrees of suprarenal insufficiency. However, exactly the opposite was found to be the case. The leeway between the production of any effect and of a fatal insufficiency is extremely slight and uncontrollable. The removal of less than three-fourths of the suprarenal tissue produces no evidence of abnormality in heat production, while the result of removing more than this bears no necessary relationship to the anatomical amount remaining. Quite uncontrollable conditions, chiefly the blood supply of the remaining fragment, determine the outcome. By this method we were not able to produce any clinical change except the typical rapidly fatal symptom-complex of suprarenal suppression. This procedure was therefore abandoned.

Ligation of Blood Vessels.—For many years the fact has been known that occlusion of the lumbosuprarenal veins would produce marked histological changes in the suprarenal gland, particularly in the cortex (8). Hartman and Blatz, in 1919, reported clinical symptoms following ligation of the lumbosuprarenal veins which were suggestive of a functional suprarenal insufficiency as their basis (9).

Ligations of the suprarenal blood vessels were carried out in the following combinations: (a) right and left lumbosuprarenal veins; (b) left lumbosuprarenal vein with excision of the right suprarenal; (c) right and left lumbosuprarenal vein with mass ligation of the collateral renal-suprarenal vessels; and (d) left lumbosuprarenal vein with mass ligation of the collateral left suprarenal vessels and excision of the right suprarenal.

In ligating the lumbosuprarenal vein the peritoneum over it was slit just proximally and distally to the suprarenal gland and a silk ligature carried around the vein on an aneurysm needle without manipulating the gland or interfering with any of its nerve supply except those filaments that run in the sheath of the suprarenal vein. After ligation of the vein, congestion and swelling of the gland were observed to different degrees, the variation of which was extreme and unpredictable. Ligation by the same method in a series of animals gave entirely different results, depending on the collateral circulation which developed.

Freezing.—This method was developed by Marine and Baumann in their experiments to cripple the suprarenal glands in rabbits. They

pointed out the difficulties encountered in freezing the right gland and the relative ease on the left side. In the main the same situation prevails in cats except that the right suprarenal is somewhat more accessible, being usually less closely adherent to the vena cava. In some cases both glands were frozen, though more frequently the left was frozen and the right excised. Mobilizing the left suprarenal gland sufficiently to freeze it is very satisfactorily accomplished. The fatty areolar tissue is dissected back with the probe point for a distance of several millimeters so that the gland can easily be elevated with most of the nerves and minute blood vessels attached. A spray of ethyl chloride reaches all parts of the surface of the cortex. The duration of the freezing varies from 15 to 45 seconds, depending upon the degree of injury desired. There was difficulty encountered in obtaining ethyl chloride tubes having a spray sufficiently fine. Bengué tubes were the best available in this country at the time, and only about one in four of these was satisfactory. During the freezing the gland is blanched, but on thawing the circulation is seen to resume at once. To freeze the right suprarenal the lumbar approach is obligatory and the peritoneum is not opened, while for the left one either the lumbar or midline incision is satisfactory, and the suprarenal is reached transperitoneally. In either case the proper exposure of the suprarenal gland, effected by a fairly deep self-retaining retractor after the field is walled off and other structures are protected by sponges, is an important factor in controlling the degree of injury.

Controls.—Two types of control experiments were performed: (a) freezing tissue around the mobilized suprarenals, and (b) excision of one suprarenal gland.

(a) Two normal animals were subjected to operation in which both suprarenals were prepared as for freezing; the tissues about them were frozen but the glands themselves were not. This procedure included all the elements of shock, general trauma, and nerve and vascular injury that obtained when the glands were frozen, except for the actual injury to the cells of the suprarenal cortex.

(b) Five animals had one suprarenal excised, nothing being done at the time to the other gland.

In experiments in which thyroid function may be intimately concerned, the use of iodine for skin sterilization is contraindicated. Chemical sterilization of the skin by an alcoholic picric acid solution was the method used in these experiments.

Presentation of Data.

35 experiments were conducted on 24 cats in which the respiratory exchange was followed before and after operation, as follows: freezing, 10; ligation, 17; partial removal, 6; operative trauma, 2. The types of reaction to subtotal suprarenal insufficiency as observed in these cats may be roughly classified in three groups (Table I):

TABLE I.

Suprarenal glands.	Group I. No rise in heat production.		Group II. Rise in heat production.		Group III. Fall in heat production.	
	No.	Per cent.	No.	Per cent.	No.	Per cent.
Frozen.....	1	10	6	60	3	30
Ligated.....	7	41	6	35	4	24
Partial removal and trauma (controls).....	7	88	0	0	1	12
Total.....	15	43	12	34	8	23

Group I. Those that live indefinitely and show no increase in basal metabolism.

Group II. Those that show a significant increase in basal metabolism (over 10 per cent).

Group III. Those that show a decrease in metabolism to a rapidly fatal termination.

There are variations in the reaction not specified in the above groups which will be referred to in the Discussion. No experiment has been considered to fall in Group II unless there was an increase of at least 10 per cent above the highest preoperative figure. The following six protocols have been selected as illustrative of the types of reaction observed.

Group I; Protocol 1.—Cat 10; adult, female (Table II). Aug. 29, 1921. Began metabolic determinations. Oct. 17. Under ether distally and proximally

ligated right and left lumbosuprarenal veins. Oct. 18. Active, eating well. Oct. 22. Normal in appearance. Oct. 24. Wound healed, stitches removed. Feb. 1, 1922. In stock; apparently in normal health.

Group II; Protocol 2.—Cat 7; adult, female (Table III). Sept. 2, 1921. Began metabolic determinations. Sept. 29. Under ether removed right suprarenal and froze the left. Oct. 1. Good appetite, alert, pupils dilated. Oct. 4. Marked diarrhea; eating very heartily (double ration). Oct. 6. Eating 150 gm. of milk and 150 gm. of meat each day and still seems hungry. Head feels

TABLE II.

Cat 10.

Date.	Weight.	O ₂	CO ₂	R.Q.	Total No. of calories.	No. of calories per kilo per hr.	Remarks.
<i>1921</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>				
Aug. 29	3,261	4.230	4.280	74	13.91	2.13	Control.
Sept. 2	3,138	3.805	3.825	73	12.58	2.00	"
" 14	3,148	4.485	4.520	73	14.86	2.36	"
" 16	3,154	3.655	3.955	79	12.21	1.94	"
" 30	3,198	3.730	4.090	80	12.49	1.97	"
Oct. 6	3,112	4.660	4.920	77	15.50	2.49	" (slightly restless).
" 10	2,972	4.065	4.250	76	13.53	2.28	"
" 13	2,818	3.860	4.050	76	12.89	2.29	"
Oct. 18	2,923	4.250	4.680	80	14.30	2.45	Oct. 17. Ligation of right and left suprarenal veins.
" 20	3,119	4.720	5.140	79	15.86	2.54	
" 22	3,181	4.400	4.750	79	14.66	2.30	
" 24	3,130	4.380	4.380	73	14.40	2.30	
" 26	3,204	4.900	5.430	81	16.43	2.56	
" 28	3,208	4.780	5.480	83	16.26	2.48	
" 31	3,185	4.540	4.870	78	15.18	2.38	
Nov. 3	3,328	4.480	5.130	83	15.22	2.29	
" 8	3,227	3.990	4.360	79	13.45	2.08	
" 11	3,060	4.230	4.310	74	14.02	2.29	
" 16	3,169	3.910	4.160	77	13.10	2.07	

warm. Oct. 27. Fur has become very glossy and smooth. Dec. 30. Sacrificed in good health.

Protocol 3.—Cat 18; three-quarters grown, female (Table IV). Sept. 26, 1921. Began metabolic determinations. Oct. 20. Under ether proximally and distally ligated right and left suprarenal veins. Oct. 22. Feels warm, eating well, active. Oct. 25. Not eating, very quiet. Oct. 26. Very weak, coughing occasionally. Oct. 27. Appetite returning. Nov. 18. Losing weight and strength, eating almost nothing, KI 25 mg. by mouth. Nov. 19. Very weak and

listless, scarcely holding up head; given emulsion of alcoholic extract residue from ox suprarenal cortex. Nov. 20. Very active and alert this morning, remarkable appetite, eating 100 gm. of meat without intermission; KI by mouth. Nov. 22. KI by mouth. Nov. 24. KI by mouth, gaining in weight, appearance improving. Nov. 25. KI 10 mg. Nov. 30. KI by mouth. Dec. 30. Sacrificed.

Group III; Protocol 4.—Cat 19; adult, female (Table V). Sept. 26, 1921. Began metabolic determinations. Oct. 7. KI 25 mg. by mouth. Oct. 29. Under

TABLE III.

Cat 7.

Date.	Weight.	O ₂	CO ₂	R.Q.	Total No. of calories.	No. of calories per kilo per hr.	Remarks.
<i>1921</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>				
Sept. 2	3,119	4.240	4.520	77	14.24	2.28	Control.
" 14	2,872	4.625	4.870	76	15.50	2.70	" (restless).
" 16	2,879	4.085	4.495	80	13.73	2.38	"
Sept. 30	2,891	3.910	4.250	79	13.12	2.28	Sept. 29. Right suprarenalectomy; left suprarenal frozen.
Oct. 2	2,784	4.410	4.480	74	14.57	2.62	
" 4	2,880	5.740	5.950	75	19.14	3.32	
" 6	3,028	5.710	5.950	76	18.94	3.13	
" 8	3,290	5.770	6.160	78	19.20	2.92	
" 10	3,242	4.920	5.080	75	16.33	2.52	
" 12	3,267	4.980	5.110	75	16.44	2.52	
" 15	3,405	5.010	5.200	75	16.72	2.46	
" 18	3,478	5.300	5.710	78	17.80	2.56	
" 20	3,675	5.760	6.250	79	19.29	2.62	
" 22	3,560	5.230	5.500	76	17.51	2.46	
" 24	3,506	4.900	5.010	74	16.29	2.32	
" 28	3,620	4.850	5.160	77	16.24	2.24	
Nov. 1	3,542	4.790	4.920	75	15.83	2.23	
" 8	3,202	3.670	3.930	78	12.25	1.91	
" 15	3,174	4.880	5.130	76	16.33	2.57	
" 21	3,444	4.560	4.670	74	15.18	2.20	

ether doubly ligated right and left suprarenal veins. Oct. 31. Very quiet and dopy in appearance. Nov. 2. Very weak, scarcely able to stand, feels cold, pupils dilated. Died during the night.

Autopsy.—Thyroid lobes very small, translucent, amber-yellow. Heart dilated, gray specks in pericardium; left suprarenal medulla is hemorrhagic, cortex necrotic. Three small, congested veins from posterior surface extending into perirenal fat and one into the renal vein. Right suprarenal is swollen but is not hemorrhagic,

TABLE IV.

Cat 18.

Date.	Weight.	O ₂	CO ₂	R.Q.	Total No. of calories.	No. of calories per kilo per hr.	Remarks.
<i>1921</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>				
Sept. 26	2,245	3.205	3.400	77	10.71	2.39	Control.
Oct. 5	2,203	3.810	4.160	79	12.84	2.91	" (temperature 16°C; shivering).
" 10	2,047	2.585	2.770	78	8.63	2.11	Control.
" 13	1,951	3.115	3.365	79	10.43	2.67	"
Oct. 21	1,945	4.220	4.430	76	14.10	3.63	Oct. 20. Ligated right and left suprarenal veins.
" 23	1,937	3.770	4.090	79	12.62	3.26	
" 25	1,910	4.160	4.400	77	13.86	3.63	
" 27	1,937	3.430	3.710	78	11.56	2.99	
" 31	1,984	3.220	3.540	80	10.81	2.72	
Nov. 2	2,014	3.480	3.800	79	11.73	2.91	
" 4	2,001	3.730	3.980	78	12.41	3.10	
" 7	2,053	3.500	3.870	80	11.82	2.88	
" 9	2,147	4.200	4.640	80	14.18	3.30	
" 11	2,132	3.780	4.120	79	12.71	2.98	
" 14	2,156	3.480	3.920	82	11.74	2.72	
" 16	2,024	2.760	2.920	77	9.20	2.27	
" 18	1,894	2.190	2.390	80	7.29	1.92	
" 21	1,935	2.710	2.920	78	9.10	2.35	
" 22	1,988	3.340	3.820	83	11.33	2.85	
" 23	2,061	3.560	4.090	84	12.08	2.93	
" 25	2,110	3.190	3.550	81	10.74	2.54	
" 26	2,096	2.790	3.260	85	9.49	2.26	
" 28	2,228	3.670	4.250	84	12.49	2.80	
" 30	2,246	3.490	3.940	82	11.80	2.63	
Dec. 2	2,182	3.210	3.660	83	10.86	2.49	
" 5	2,077	2.530	2.760	79	8.52	2.05	
" 7	2,052	2.540	2.710	78	8.45	2.06	
" 9	2,022	2.450	2.670	79	8.24	2.04	
" 12	2,048	3.120	2.990	70	10.19	2.49	
" 14	2,090	2.730	3.120	83	9.26	2.21	
" 16	2,130	3.010	3.320	80	10.14	2.38	
" 20	2,214	3.240	3.770	85	10.98	2.48	

medulla appears intact grossly and microscopically, cortex yellowish white in color, microscopically necrotic. Abundant subcutaneous and abdominal fat. Other organs normal.

Protocol 5.—Cat 8; adult, female (Table VI). Aug. 29, 1921. Began metabolic studies. Sept. 29. Under ether removed right suprarenal and froze left. Sept. 30. Ate well. Oct. 1. Quiet and drowsy. Oct. 4. Very quiet, appears

TABLE V.

Cat 19.

Date.	Weight.	O ₂	CO ₂	R.Q.	Total No. of calories.	No. of calories per kilo per hr.	Remarks.
<i>1921</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>				
Sept. 26	2,089	3.245	3.515	79	10.85	2.59	Control.
Oct. 3	2,158	2.800	3.200	83	9.49	2.20	"
" 6	2,125	2.830	3.260	84	9.58	2.25	"
" 11	2,148	2.560	2.800	80	8.55	1.99	"
" 14	2,264	2.730	3.270	87	9.35	2.07	"
" 19	2,289	3.240	3.550	80	10.85	2.37	"
" 21	2,303	3.450	3.710	78	11.58	2.51	"
" 25	2,152	2.600	2.760	77	8.69	2.02	"
Oct. 31	2,040	2.410	2.380	72	7.91	1.94	Oct. 29. Ligated right and left suprarenal veins.
Nov. 2	1,938	1.150	1.350	85	3.93	1.01	Nov. 3. Died.

TABLE VI.

Cat 8.

Date.	Weight.	O ₂	CO ₂	R.Q.	Total No. of calories.	No. of calories per kilo per hr.	Remarks.
<i>1921</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>				
Aug. 29	2,412	4.135	4.320	76	13.75	2.85	Control.
Sept. 2	2,394	4.210	4.285	74	13.93	2.91	"
" 14	2,599	4.180	4.540	79	14.01	2.70	"
" 16	2,581	4.440	4.700	77	14.80	2.87	"
Sept. 30	2,719	3.530	3.980	82	11.92	2.19	Sept. 29. Right suprarenalectomy; left suprarenal frozen.
Oct. 2	2,649	4.400	4.510	75	14.51	2.74	
" 3	2,594	4.450	4.720	77	14.87	2.87	
" 6	2,502	3.080	3.240	77	10.21	2.04	
" 8	2,440	2.050	2.310	82	6.92	1.42	

dopey, alopecia, refuses meat, took 130 cc. of milk. Oct. 6. Lactose added to the milk; eating very little now. Oct. 7. Extremely weak. Lactose and milk 20 cc. by pipette. Oct. 8, p.m. Died.

Autopsy.—Abdominal and subcutaneous fat well preserved. Heart, normal color; heart's blood not coagulated. Right suprarenal absent; left embedded in omental adhesions, shows marked areas of necrosis in the cortex, medulla normal histologically. Stomach empty. Numerous erosions and hemorrhagic areas in mucous membrane.

Control. Protocol 6.—Cat 25; adult, male (Table VII). Oct. 28, 1921. Began metabolic observations. Nov. 5. KI 25 mg. by mouth. Nov. 8. Under ether mobilized both suprarenals and froze the tissue around them including blood vessels and nerves. Nov. 9. Normal appearance, eating well. Nov. 13. Normal behavior, marked dermatitis over shaved areas. Nov. 22. Wounds completely healed. Cat appears normal in every way.

TABLE VII.

Cat 25.

Date.	Weight.	O ₂	CO ₂	R.Q.	Total No. of calories.	No. of calories per kilo per hr.	Remarks.
1921	gm.	gm.	gm.				
Oct. 28	2,589	3,790	3,780	73	12.43	2.40	Control.
" 31	2,683	4,710	4,750	73	15.62	2.91	"
Nov. 3	2,880	3,990	4,630	84	13.61	2.36	"
" 8	2,955	5,280	5,850	81	17.70	2.99	"
Nov. 9	2,785	4,540	4,640	74	15.09	2.71	Nov. 8. Control freezing operation.
" 11	2,618	3,780	3,960	76	12.60	2.41	Marked dermatitis.
" 13	2,612	4,470	4,870	79	15.03	2.88	
" 15	2,570	3,600	4,000	81	12.10	2.35	
" 17	2,522	3,400	3,790	81	11.46	2.27	
" 19	2,562	3,500	3,710	77	11.69	2.28	
" 21	2,574	3,780	4,100	79	12.65	2.46	
" 23	2,571	3,530	3,920	81	11.86	2.31	
" 26	2,619	3,800	4,260	82	12.82	2.45	
" 30	2,888	4,870	5,030	75	16.13	2.80	
Dec. 13	2,834	3,900	4,600	86	13.28	2.34	

Group I.—In this group the animals survive indefinitely and there is no increase in heat production of over 10 per cent. Eight, or 30 per cent of the twenty-seven experiments following freezing or ligation, presented this type of response to operation. These usually showed no noteworthy symptoms. Within 24 hours they had regained a normal appetite and behavior. In two cases there was a decrease in heat production lasting 2 weeks or more, not leading

to the death of the animal and with a subsequent return to the normal rate. Examination of the condition of the suprarenals either at autopsy or at a subsequent operation showed large areas of uninjured cortex, on both gross and microscopic examination. The medulla appeared quite normal. It is worthy of note that only one cat out of a total of ten, in which the suprarenals were frozen, fell in this group, and in this one the dissection of the gland for freezing was unsatisfactory on account of an excessive number of collateral blood vessels—a result of a previous ligation; while following vessel ligation, seven of seventeen showed no significant change in heat production. In the latter there was a well developed collateral circulation to the renal vein, the perirenal vein, and the diaphragm.

Protocol 1 illustrates this type of reaction. Eight determinations of the basal metabolism had been made over a period of 45 days, with a maximum variation of + 14 per cent and - 11 per cent from the average of 2.18 calories per kilo per hour. Both suprarenal veins were ligated proximally and distally to the suprarenal gland. Following this the gaseous metabolism was determined eleven times in 30 days—every other day for the first 2 weeks after operation. At no time was there a significant departure from the preoperative range, the maximum increase being 3 per cent above the highest normal figure.

Group II.—This group includes those cats whose heat production following suprarenal injury is increased at least 10 per cent above the highest preoperative figure. Twelve, or 44 per cent of the twenty-seven freezing or ligation experiments, showed this type of reaction. The increase varied from 18 to 44 per cent above the preoperative average and from 10 to 30 per cent above the highest preoperative figure. With one exception the rise in basal metabolism lasted from 9 to 38 days before again reaching the preoperative level. The increase in heat production was absolute as well as relative and in every case the cats survived the effect of suprarenal insufficiency indefinitely.

Coincident with the increased basal metabolism there were clinical manifestations of improved nutrition. Similar manifestations have been reported by Marine and Baumann in rabbits. The surface temperature seemed increased; the hair, which was shaggy in-

mediately after operation, became very sleek; and the appetite was often greatly increased. In spite of a food intake of double the normal amount the weight frequently was maintained at a level below its preoperative normal, though in some cases the cat gained tremendously in weight. Diarrhea was frequently observed, usually occurring at the height of the increase in heat production. Respiration was increased in rate and depth and at times there was a marked change in attitude and behavior, alertness and restlessness being observed.

Protocols 2 and 3 show typical examples of this type of reaction after freezing and ligation respectively. Cat 7 had the right suprarenal removed and the left frozen. The heat production was increased 36 per cent, reaching its maximum on the 5th day after operation and being maintained above the preoperative average for 20 days. The appetite was greatly increased and the cat gained 900 gm. in weight during this period of increased heat production, even gaining in weight when its basal metabolism was + 30 per cent. The total heat production also was increased by 30 per cent even before this gain in weight occurred. Diarrhea was noted on the 5th day after operation. The wounds healed promptly and the fur became very smooth.

Following this phase of increased heat production a slight depression averaging 10 per cent below the normal usually occurs, lasting from 1 to 4 weeks. That this may be partly due to an exhaustion of the thyroid gland is suggested by two facts. Potassium iodide given in two cases during this period of depression was followed by a prompt return to the normal level. And in several instances the thyroid gland was found 30 days after saturation with potassium iodide to be markedly hyperplastic in cats which had shown an increased basal metabolism after suprarenal crippling. At the time of being sacrificed the heat production had become subnormal. In control animals no such rapid exhaustion of the thyroid was observed.

Cat 18 showed an increase in heat production reaching the maximum of 44 per cent on the 1st and 5th days after ligation of both suprarenal veins. 2 days after operation it had regained its appetite and activity; however, by the 5th day after the operation it had become very inactive, was weak, and had lost its appetite. Its general

condition gradually improved; the basal metabolism remained elevated for 25 days, on the 20th day after the operation being $+ 40$ per cent. At this time the animal lost its appetite and became very asthenic, rapidly losing weight; the heat production fell to 20 per cent below the preoperative figure. KI was given on November 18; on November 19 it ate less than 10 gm. of meat and did not hold its head up. Emulsion of suprarenal cortex extract was given by pipette. On November 20 the animal was very active, running and jumping, and when given food ate 100 gm. of meat without pause. The basal metabolism rose continuously for 3 days to a point above the preoperative range, it then became subnormal for a week, after which it resumed the original level. At autopsy the right suprarenal was found markedly atrophied; from the left there had developed a collateral circulation to the renal vein and the perirenal tissue. Colloid had disappeared almost completely from the thyroid vesicles and the epithelium was hypertrophic in spite of the fact that the cat had received potassium iodide 30 days previously in several doses.

Group III.—In this group the metabolism decreases slowly or rapidly till death. Seven, or 26 per cent of the twenty-seven animals, reacted in this way after freezing or ligation. The decrease in metabolism is on the average about 25 per cent. In the fulminating cases the picture is exactly like that after excision of both suprarenals (10), and the animal dies in 2 to 5 days. Two of this group lived 9 and 13 days respectively.

Histological examination of the suprarenal glands showed in each case marked injury to the suprarenal cortex. After freezing, the medulla had quite a normal appearance except for some congestion; its cells maintained their chromophilic properties. After ligation there was often very extensive hemorrhage into the medulla, though in some cases it remained entirely uninvolved. But the cortex always showed extensive necrosis in the animals of this group after either freezing or ligation. In some instances the entire cortex appeared to be necrotic, in others some areas of cortical cells staining fairly normally were found.

One cat in which fully seven-eighths of the suprarenal substance was excised reacted in this manner, showing marked reduction in heat production, and died $2\frac{1}{2}$ days after operation. No viable supra-

renal cells could be found, and the vein leading from this remnant was completely thrombosed.

Protocols 4 and 5 are illustrative of this type of reaction. Cat 19 showed a decrease of 54 per cent 4 days after ligation of both suprarenal veins, and was unable to walk. It died the following night. Autopsy revealed hemorrhage into the medulla of the left suprarenal and marked necrosis in the cortex. Three small congested veins extended to the perirenal fat and one to the renal vein. The right suprarenal showed no hemorrhage into the medulla, which seemed normal, while the cortex showed large areas of necrosis. Cat 8 lived 9 days after excision of the right suprarenal and freezing of the left. The day after operation the heat production was 22 per cent below normal, it then rose on the 3rd and 4th days after the operation to the upper part of its normal range, thereafter falling sharply to 50 per cent below normal on the 9th day, and death occurred that night. There was increasing weakness so that on the 8th day the cat could not stand and anorexia was marked, though for the first 5 days it ate over 100 gm. of milk and some meat each day. Autopsy showed well preserved fat stores, right suprarenal absent, left suprarenal cortex with large areas of necrosis, and medulla with normal staining properties.

The animals suffering from a fatal suprarenal insufficiency, though living over a week, give definite evidence of cardiac weakness. The heart rate may become irregular, the mucous membranes are somewhat cyanotic, and the animal goes into collapse on slight exertion, which speaks strongly for a marked diminution in its cardiac reserve. At autopsy the heart is somewhat dilated and the musculature, especially of the right ventricle, is flabby.

Controls.—In the two cats in which both glands were mobilized and the tissues about the glands were frozen, the heat production after operation showed no increase. Also the five animals in which one suprarenal was removed showed no increase of 10 per cent above the preoperative level. That these animals could, however, show the typical rise in basal metabolism was demonstrated in three of these five cats by either a previous or subsequent operation.

DISCUSSION.

The difference in reaction to suprarenal insufficiency in cats and rabbits, as reported in the literature, seems now to be cleared up. The previous failure to observe the characteristic increase in heat production in cats was due to the masking of this by the more profound effect of entire suppression of suprarenal function. In the six cases in which death resulted within a week from the suprarenal insufficiency, the heat production was invariably found to be continuously diminished, while in the two in which the suprarenal injury caused death, though the animal lived over a week, heat production in one case on the 4th day reached its highest preoperative level, and in the other case on the 5th day exceeded it, being followed in each case by a fall to death. Marine and Baumann observed in a small percentage of rabbits, also, this decrease in heat production to a rapidly fatal termination, and in them no accessory suprarenals could be found. It seems established then that when the suprarenal insufficiency approaches closely the suppression of suprarenal function heat production falls and death rapidly ensues. This is apparently due to the fact that the suprarenal glands, either by addition or subtraction, influence the reactive medium of the body in some manner essential to the continuance of vital metabolic processes.

Suprarenal injury less severe than the above causes in the majority of cats a profound effect upon the calorific mechanism, characterized by a significant increase in heat production. Of the twenty cats indefinitely surviving the suprarenal injury, twelve, or 60 per cent, showed this rise in basal metabolism, while of the control seven experiments involving all the elements of traumatization and manipulation of the suprarenals without serious damage to the glands, in none did the basal metabolism vary from its normal range by 10 per cent. In every case evidence of an increase in heat production was present in the 1st week, though the maximum frequently was not reached until later—24 days after operation in one instance. The rise lasted on the average 18 days before again reaching the pre-operative base-line.

The secondary waves in the heat production curve mentioned by Golyakowski as occurring in dogs, and by Marine and Baumann in

rabbits, were very well marked in several instances. Cat 18 illustrates this phenomenon, one peak being in the 1st week after operation and another in the 3rd week.

After a period of suprarenal insufficiency associated with a significant rise in heat production there is usually a fall to 10 per cent or more below the base-line. That this is not altogether the effect of long training is shown by the fact that if it is followed sufficiently long the normal rate is resumed. This phenomenon resembles the period of hypothermia which is frequently seen in the convalescence from any prolonged fever. That thyroid exhaustion may be partly responsible for it is suggested by the effects of KI and by the rapid production of hyperplasia of the thyroid in these animals.

Of the three methods for producing suprarenal insufficiency in cats, partial excision was entirely unsatisfactory. Vein ligation gave good results in some cases but the collateral circulation was a dominant factor in the result which could not be predicted or controlled. In our hands freezing offered the most satisfactory means of controlling suprarenal injury.

In addition to the effect on heat production there are clinical symptoms, some of which can be readily correlated with it. Increased appetite, which may or may not be associated with a gain in weight, sleekness of fur, and rapidity of wound healing are nutritional manifestations which can be readily explained on the basis of thyroid function. There are other phenomena, however, which suggest vastly wider interrelationships between the suprarenal gland and the body tissues. In fatal suprarenal insufficiency gastric ulcer was frequently observed in this series in confirmation of the observations of other workers (11). Also symptoms of disturbed gastrointestinal function, most often manifest as diarrhea, were usually evident during the period of suprarenal insufficiency, most frequently observed at the time of maximal increase in basal metabolism. In one case diarrhea with numerous small blood clots was noted. Furthermore, the profound asthenia of the animals developing in the cases of fatal suprarenal insufficiency bespeaks a fundamental function of the suprarenal in relation to some part of the nerve-muscle motor mechanism. That this is not due merely to restriction of food intake was shown by the two cats which survived a fatal in-

sufficiency for over a week. In both cases operative recovery was prompt but a secondary profound weakness manifested itself about the 4th day which was well in advance of the stage of marked reduction in food intake. Cat 8, for example, was so weak, a week after operation that it could not walk on a flat surface, yet rough measurement showed the food intake up to this time to be 75 per cent of the caloric requirement of the animal. Rapid depletion of the glycogen stores have been demonstrated by previous observers (12). And other metabolic disturbances apart from the mobilization and use of fat were suggested by the rapidity of emaciation and the extreme degree to which it may be carried without exhaustion of the fat depots. Cat 12, for example, lost one-fifth of the body weight in 13 days, yet had abundant subcutaneous and mesenteric fat at autopsy. Extensive studies of the protein and especially of the water metabolism would be necessary to clear up this phenomenon.

A great mass of literature has sprung up about clinical suprarenal insufficiency, all of it vague, indefinite, and, up to the present, without any scientific support in fact (4), as there has been no method of differentiating the degrees of sublethal suprarenal insufficiency. Comparative measurements of heat production in the presence of an iodized thyroid may possibly offer a means of following to some degree the various phases of insufficiency of the suprarenal cortex. This work with cats, as well as that of Marine and Baumann with rabbits, shows that the partial suprarenal cortex insufficiencies produced experimentally are transitory and that compensation takes place rapidly or the animal dies. Therefore, one would not expect to demonstrate a suprarenal insufficiency in animals that have survived the injury for months.

Further evidence of the importance of the cortex as opposed to the medulla in influencing essential tissue functions is indirectly afforded by these experiments. In all cases in which death ensued from the suprarenal insufficiency, whether induced by freezing or ligation, marked damage to the cortex was evident, while the medulla usually retained its normal appearance grossly and microscopically. Even though secretion of epinephrine is possibly suppressed by freezing, vessel ligation leaves the nerve supply which controls this secretion, for the most part intact. There is a path for its discharge—

a definite collateral circulation between the suprarenal medulla and the perirenal tissue having been reported by Cow in 1914 (13) after ligation of the suprarenal vein proximally and distally to the gland. The collateral venous circulation of the suprarenal becomes markedly increased if the animal survives. These enlarged veins can readily be traced to the perirenal tissue, not infrequently to the diaphragm, and usually one or two directly to the renal vein. If it could be shown that under these conditions epinephrine was still discharged, it would necessarily follow that this agent played no essential part in the increased heat production.

It is impossible to study the syndrome of suprarenal insufficiency in cats without being impressed by the points of similarity between it and some of the outstanding features of Graves' disease. The most prominent single phenomenon in each is a significant increase in heat production. Each has a phase of asthenia and exhaustion, with evidence of severe cardiac damage. Diarrhea and nutritional and weight changes are common to each. Hyperplasia of the thyroid is produced in each and is characteristic of neither. Marine and Baumann have shown that the thyroid is indispensable for the increased heat production following suprarenal injury in rabbits but not for the asthenia and fatal termination. And, finally, that rather unusual form of Graves' disease running a rapidly fatal course in a very few weeks with severe asthenia, prostration, and coma bears a striking resemblance to the effect of fatal suprarenal insufficiency when death is postponed for several days.

CONCLUSIONS.

1. Severe and sufficient non-fatal injury to the suprarenal cortex by freezing or by ligation in cats causes a significant and prolonged increase in heat production.
2. Lethal injury to the suprarenals by freezing, ligation, or partial excision in cats causes a fall in heat production.
3. Insufficient injury to the suprarenals by freezing, ligation, or partial excision in cats produces no significant alteration in heat production.
4. Further evidence of a close thyroid-suprarenal cortex interrelationship is indicated by the rapidity of thyroid hyperplasia and by the effects of KI after suprarenal crippling.

I wish to express my appreciation for the continuous advice and interest of Dr. David Marine throughout this investigation.

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THE EXPERIMENTAL PRODUCTION OF PERIARTERITIS NODOSA IN THE RABBIT WITH A CONSIDERATION OF THE SPECIFIC CAUSAL EXCITANT.

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PLATES 6 TO 14.

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In the early reports (1) of periarteritis nodosa there occurs considerable discussion as to whether or not the lesions constitute a disease *per se* or represent a peculiar invasion of the vascular structures either by the "virus of syphilis" or by certain known microorganisms. The subsequent opportunities of study, however, have as a whole established it as a specific infectious disease of unknown etiology exhibiting a selective affinity for the vascular system, to which the essential lesions are confined. The disease is characterized especially by entrance of the injurious agent into the periarterial tissues, and the adventitia and media of the smaller arteries, leading to acute exudation and subsequent degenerative changes. The process may extend in the intima, causing rupture of the elastic lamina and the formation of multiple true aneurysms, extensive thrombosis or actual vascular rupture with hemorrhagic extravasation, or false aneurysmal formations. Moreover, signs of chronic inflammation associated with proliferative or reparative changes are present in certain of the vascular lesions.

The literature on periarteritis nodosa contains descriptions of the pathology and of the symptoms of the disease and little reference to the etiology regarding which the main reference is to syphilis. *Treponema pallidum* has been looked for diligently in tissues stained by the Levaditi method, but in no instance has it been detected. Other bacteria too have usually not been found. It is true that Babes and Mironescu (2) described diplococci and streptococci in the arterial lesions, but they report also an instance of syphilitic aneurysm of the aorta in which diplococci were detected. Although cited as a demonstration of the occurrence of streptococci in the lesions, these authors apparently do not regard the condition of periarteritis nodosa as present

in their specimens. Longcope (3) observed "a few groups of cocci in chains" in specially stained preparations of the kidney tissue distinct from the nodules; and absent from other involved organs.

The bacteriological investigations included blood cultures made during life and cultures at autopsy from the heart, pericardial cavity, gall bladder, peritoneal cavity, and the nodular lesions themselves. Beitzke's (4) *intra vitam* blood cultures were negative. Baehr's (5) and Manges and Baehr's (6) aerobic and anaerobic cultures made from blood during life and nodules excised at operation were also negative. Lamb (7) studied two cases and isolated various microorganisms from different organs and a streptococcus from a nodule. He inoculated, with negative results, with portions of the nodules and the streptococcus a number of guinea pigs, rabbits, and a monkey. Klotz (8) studied two cases and obtained *Staphylococcus aureus*, *B. proteus*, streptococci, and a diphtheroid bacillus from the heart's blood, bile, subcutaneous nodules, and the liver. He injected streptococci about the periarterial tissues or lymph spaces and induced certain lesions which he specifically states were not those of periarteritis nodosa.

The observations of von Haun (9) are more important. He injected intraperitoneally into two guinea pigs the blood of a proven case. The animals showed no evidences of the disease but died after 8 weeks. The heart's blood of these animals was injected into other guinea pigs and an organ emulsion, prepared from these animals, was injected into other guinea pigs. The second series of guinea pigs showed microscopic changes in the smaller arteries of organs and a disseminated inflammatory lesion of the adventitia and media of different arteries. Von Haun concludes that periarteritis nodosa is a specific infectious disease due to an unknown etiological factor present in the circulating blood and capable of inducing inflammatory changes in the walls of the blood vessels of guinea pigs.

Human Material.

The material employed in the present study was derived from a typical case of periarteritis nodosa admitted into the Charity Hospital of New Orleans on March 28, 1921. The patient was a colored male laborer, 32 years of age, a native of Louisiana. He had become ill 1 week before admission, suffering from general weakness, pain in the epigastric area and lower abdomen, and vomiting. The general physical examination with the exception of the abdominal tenderness was negative. The temperature chart showed an occasional rise above normal from 99–100°F. The pulse ranged from 80 on date of admission to 112 shortly before death. The respirations varied from 20 to 32. The Wassermann reaction was negative. The leucocytic counts ranged from 10,000 to 16,600 and the neutrophils from 71 to 80 per cent. Only one urinalysis is recorded, which was negative.

Radiological examination of the alimentary tract showed hyperperistalsis of the stomach, and pyloric spasm and hypermotility of the duodenum. Death occurred on April 29.

The autopsy was performed 72 hours after death. The body had been kept in the refrigerator at approximately 0°C. Only the anatomical structures showing pathological changes are described.

Peritoneal Cavity.—The coils of the small intestine are found adhered on the right side to a mass about the size of a coconut, consisting of a blood clot surrounding the right kidney. Another large clot is present beneath the omentum, and numerous more recent smaller clots between the coils of the intestines.

Stomach.—The gastropiploic artery presents two large fusiform aneurysmal dilatations measuring respectively on cross-section 1.5 and 2 cm. in diameter. At about the middle of this vessel is a small branch which also shows a small nodule. In two of the nodules on cross-section there is a more or less pale red crescentic mass which on serial sectioning becomes smaller and finally disappears as the normal portion of the vessel is reached.

Kidneys.—When the previously described large blood mass is removed, the source of the hemorrhage can be traced to a ruptured aneurysmal sac within the outer portion of the cortex of the right kidney. The external surface of this kidney presents multiple bizarre shaped areas of mottled red and buff color. These areas are composed of arboriform injected vessels, some of which have ruptured, giving rise to petechial extravasations. Some areas contain pale centers and suggest infarcts. The sectioned organ (Fig. 1) shows extensive pathological changes. Numerous more or less rounded nodules from cream-white to garnet color and varying from 2 mm. to 1.5 cm. in size are found scattered throughout the kidney but are most prevalent in the cortical portion and the columns of Bertin. Upon serial section they terminate abruptly in the original vessel from which they arise and which in certain instances is so small as to be visible with difficulty or only with the hand lens. With the exception of the blood clot, the left kidney presents the same condition but to a less degree.

Heart.—The heart weighs 480 gm. Scattered irregularly along the course of the coronary arteries and their rami are found multiple

discrete nodosities varying in size from 2 to 6 mm. which present the appearance of partially filled pea-pods. They are for the most part of a yellow or tan color but a few are mottled with deep red and still others are entirely red. Upon section the nodules show irregular lamellations of dirty white and red color. Like those of the kidney they terminate abruptly and in some instances the vessel with which they are connected is found with difficulty.

Brain.—There are present a few small areas of sclerosis in the vessels of the circle of Willis. Neither these nor the other vessels of this organ, however, show the nodular changes described in the other organs.

Microscopic Examination.

Besides the structures presenting gross lesions, certain arteries in the liver and afferent arteries to the mesenteric lymph nodes present the microscopic changes of periarteritis nodosa.

The lesions may be classed into three groups: (1) the inflammatory reaction, or injury present in the vessel walls; (2) the resultant vascular lesions arising from those of the first group, namely aneurysms both true and false, thrombosis, and actual vascular rupture with hemorrhage; and (3) retrograde lesions occasioned in the parenchymatous structures or other tissues through the impairment of blood supply of these parts chiefly because of the lesions of the second group.

In the first group the changes are present especially in the adventitia and media. Varying appearances are observed. In some vessels they are purely periarterial and adventitial lesions showing polymorphonuclear neutrophils and fibrin. In other arteries this process is associated with a zone of necrosis in the media, forming at times a complete ring extending well down to the intima with fragmentation of the elastic membrane. In still other vessels the acute cellular and fibrinous exudate extends through the media down to the subendothelial layer (Figs. 2 and 3). At times the cells in the outer vessel area consist of lymphoid or plasma cells and eosinophils. In still other vessels cellular elements are absent and only necrosis of the media is found. Occasionally in these various lesions detachment of the endothelial cells or proliferation of these cells or of the subendothelial structures is noted. The proliferative processes are, however,

especially present in the dilated arteries in which thrombosis and aneurysm formation have occurred. The lesions of the second group resulting from those of the first group consist of aneurysms both true and false, and thrombosis and hemorrhagic extravasations. In them the inflammatory cellular elements are usually absent but when present they consist especially of lymphoid and plasma cells. The third group is formed by the lesions of the tissues resulting from the previous pathological processes. They are all of retrograde character and dependent upon the deprivation of the blood supply or the result of pressure from the hemorrhagic outbursts. Different kinds of degeneration, including necrosis with karyorrhexis or karyolysis, are found in the structures in which interference with the blood supply has been produced; and numerous infarcts of different sizes occur.

The gross and microscopic lesions demonstrate the existence of the peculiar pathology of the arteries characteristic of periarteritis nodosa. From the gross standpoint, the greatest intensity of the process is in the vessels of the kidneys. The branches of the renal arteries present extensive aneurysmal formations and multiple thrombosis (Fig. 1). The rupture of one of the renal aneurysms through the cortical substance and capsule permitted the extensive hemorrhage, the immediate cause of the death of the individual. The proportionately large size of the aneurysmal dilatation of even the very small arteries, for example the vasa recta, is very striking; from a microscopic artery an aneurysm 1.5 cm. in diameter may ensue. Very large nodules are also present in the gastroepiploic artery. These lesions are more fusiform in shape than the nodules in the other situations. The lesions of the coronary arteries and their branches are multiple but are more uniform and smaller in size. The liver while presenting no gross pathological change shows lesions of the first group present in a few of the interlobular arteries. Microscopic changes are also present in certain of the arteries supplying the mesenteric lymph nodes.

Transmission Experiments.

Experiment 1.—After searing the organs the gross lesions of the kidneys consisting of several nodules of various types and sizes were dissected out with sterile instruments and fragments cultured in plain, glycerol potato blood, dextrose agar, and Löffler's blood serum. No growth was detected after 1 week. Portions of the nod-

ules were ground in a sterile mortar in sterile normal saline solution and after the coarser particles sedimented out, 2 cc. of the finer emulsion were injected into the ear vein of two full grown rabbits, A and B. Rabbit A died 2 months later.

Autopsy of Rabbit A showed gross lesions in the liver only. They consisted of a hemorrhagic nodule on the under surface, measuring 2 mm. in diameter, and of several diffuse hemorrhagic extravasations beneath Glisson's capsule and extending into the organ. Two small infarct-like areas were noted.

Under the microscope the lungs show thinning and rupture in places of the alveolar walls and a marked thickening of the musculature of certain arteries with here and there a perivascular invasion of cells of the lymphoid and eosinophilic types which affect only one portion of the vessel's circumference and extend slightly into the adventitia.

The liver shows marked accumulations of lymphoid, polymorphonuclear, and eosinophilic cells in the portal spaces, chiefly about the interlobular artery penetrating to the media, and within the connective tissue. Inside the lobules are occasional smaller clusters of polymorphonuclear neutrophil and lymphoid cells. In certain of the affected arteries the walls show degeneration of the media and dilatation and thrombosis formation (Fig. 7). The cells of the liver itself show parenchymatous and fatty degeneration.

In the heart an occasional artery is to be seen with extensive necrosis of the outer portion of the media in which are a few polymorphonuclear neutrophilic and lymphoid cells. Fatty degeneration of the myocardium also occurs.

Aside from marked cloudy swelling the kidneys show no changes.

Rabbit B was killed 18 days later after drawing blood from the heart for inoculation and cultural purposes. The aerobic, anaerobic, and Smith tissue cultures remained sterile. Only the liver shows a few small hemorrhagic extravasations to the naked eye.

Under the microscope, on the other hand, the arteries of the lung show marked hypertrophy (Fig. 8) and, in some, proliferation of the subendothelial layers producing nodules which protrude into the lumen. The adventitia about certain arteries contains cells, chiefly eosinophilic (Fig. 8) and fewer lymphoid and plasma cells.

The portal tracts show accumulations of lymphoid cells and proliferation of connective tissue cells, especially marked about the arteries although occurring diffusely also (Fig. 9). The media of some vessels is degenerated and the vessels are ruptured, leading to hemorrhage into the tissues (Fig. 13).

Experiment 2.—Portions of the kidneys, liver, and heart of Rabbit B were removed, macerated with sterile normal salt solution, and 2 cc. of the supernatant fluid were injected into the ear vein of two full grown rabbits, C and D.

Rabbit C died 1 month after the inoculation. At autopsy only an occasional hemorrhage is seen in the liver.

Under the microscope the lung arteries show dense nodules of polymorphonuclear neutrophil cells situated especially in the adventitia, some of which extend into the media (Fig. 6). In certain vessels destruction of the outer coat and media and cellular infiltration extending to the intima are present.

Rabbit D died 6½ months after the inoculation. At autopsy no gross lesions are found.

Microscopic examination of the lungs shows some arteries with greatly hypertrophied walls about which are a few lymphoid cells.

In the liver the interlobular arteries show necrosis of the media, dilatation, and cellular invasion of lymphoid cells. The portal veins in some areas are dilated and surrounded with hemorrhages.

The kidneys show congestion of vessels and exudation of serum between Bowman's capsule and the glomerular tufts.

Experiment 3.—Rabbit E was injected into the ear vein with 2 cc. of a filtrate prepared from the organs of Rabbit B of Experiment 1 killed 2 months and 18 days after inoculation with the human nodules. The filtrate was prepared from the liver, spleen, and kidney, the suspension being passed through a Berkefeld filter N. This rabbit died 3 months later and showed the most typical lesions of all the animals studied.

The lungs present small hemorrhages, on the pleural surface especially. In the right lung are two round nodular masses of a deep red color (Figs. 4 and 5). One of these nodules, measuring 4 mm. in diameter, is protruded from the anterior surface, while the other, measuring 3 mm., is located on the posterior surface. They show no intermediate connection. Near the hilum of the left lung are two small gray glistening nodules measuring approximately 1 mm.

The vessels of the heart were followed throughout their course, but no lesions were visible to the naked eye.

The surface of the liver beneath the capsule shows several small irregular hemorrhages. On section the organ shows small dilated vessels containing thrombi here and there.

The pulmonary arteries show under the microscope striking lesions. In some the adventitia contains many polymorphonuclear neutrophils and in others the infiltrating cells extend into the intima, and necrosis of the media (Figs. 10 and 11) is present. In many other arteries there occur also nodular and circumferential periadventitial accumulations of neutrophils, eosinophils, and lymphoid and plasma cells which often extend as far as the intima (Figs. 14 to 17). Rupture of the artery in the human case leading to false aneurysmal formation (changes of the second group) is represented by these lesions which are evident to the naked eye (Figs. 4 and 5). Pneumonic inflammation of the organ is absent.

In the liver the interlobular branches of the hepatic artery exhibit varying lesions. As compared with the human case we can distinguish of the first group polymorphonuclear neutrophilic and lymphoid cells and degeneration and necrosis of the media. Of the lesions of the second group the organ shows necrosis with dilatation of the arterial wall with aneurysm formation and rupture and hemorrhage (Fig. 12). Of the lesions of the third group are noted degeneration and necrosis of areas adjacent to the aneurysm and hemorrhage.

The arteries of heart and of the kidneys show occasional periarterial adventitial nodules.

Experiment 4.—Rabbit F inoculated intravenously with 1 cc. of defibrinated blood from Rabbit B of Experiment 1 has survived thus far for 6 months and is still under observation.

A consideration of the experiments, excluding the incomplete one. No. 4, shows that gross lesions consisting of aneurysms and hemorrhages occurred in the liver and lungs of many of the rabbits. Those most typical (Figs. 4 and 5) appeared in the lung of a rabbit injected with a filtrate obtained from an animal previously injected with the emulsion of the human nodules. In all the rabbits microscopic changes of some degree of intensity resembling those occurring in periarteritis nodosa of man existed. They consisted of infiltrations of the adventitia and media and even of the intima with cells of different types—neutrophils, eosinophils, and lymphoid and plasma cells. Coincidentally, degeneration and necrosis of the media, dilatation of the vascular lumen, and thromboses occurred. The intima showed proliferative changes. The veins were only exceptionally affected.

DISCUSSION.

Von Haun (9) produced injury to the arterial walls in the guinea pig by injecting blood from a case of periarteritis nodosa during life, thus demonstrating the presence of an active agent even though culture studies of the blood yielded negative results. Von Haun did not pass the blood through a filter, but he observed that the effects in animals of the second series were greater than in the first, and the inoculations proved fatal in them in 2 to 3 months. Our most pronounced results also were obtained with a second series of rabbits. The filtrate employed for inoculation yielded no microorganism. Hence the active agent may be regarded as a filter passer—through Berkefeld filter N.

The disease periarteritis nodosa, because of its rarity and infrequent recognition during life, has afforded a small field for experimental study. Among 54 reported cases, less than one-fourth record evidences of search for an etiological agent, and among these in only four instances, including the present case, has transmission of the disease to animals been attempted. However, the hardened tissues have afforded opportunity for the application of special stains for bacteria and *Treponema pallidum*; but all cases so studied have given

negative results. Although *Treponema pallidum* has been excluded the majority of observers have concluded that the affection is an infectious disease. Indeed, it seems unlikely that syphilis, which occasions such frequent vascular injury, should so rarely present the lesions characteristic of periarteritis nodosa. We believe that the experiments reported in this paper constitute additional evidence for eliminating *Treponema pallidum* as the inciting cause of periarteritis nodosa.

The pathological character of the gross and microscopic lesions suggests a specific inciting agent. It is probable that the microorganism, aside from its selective affinity for the vessel walls, produces an injury of a nature such as to induce the formation of aneurysms and thrombi. Other vascular lesions occurring in man and produced experimentally in animals by various known microorganisms do not present these characters; of them many are exudative, degenerative, and proliferative in nature, but the peculiar pathology of periarteritis nodosa, so clear-cut and readily discerned, is wanting. Thus multiple mycotic aneurysms (10), Charcot's multiple miliary aneurysms of the cerebral arteries, and various infectious lesions of the arteries (11) have no real analogy to periarteritis nodosa; while the arterial lesions produced in animals with the streptococcus and other microorganisms by Klotz (8, 12), Boinet and Romary (13), and others are distinct from those of periarteritis nodosa. Duval (14) described certain vascular changes in the arteries of rabbits due to *Bacillus mallei*, but neither thrombosis nor aneurysm occurred. In experimental typhus fever in the guinea pig Olitsky (15) described a "periarteriolitis nodosa" previously observed by Nicol which is of interest here in that hemorrhagic extravasations arise and the etiological agent is said to be filterable.

Various opinions have been held as to the manner of invasion of the microorganism in periarteritis nodosa. As our inoculations were made intravenously, the infectious agent was placed directly into the blood stream. Hence it appears probable that it is introduced in the outer vascular coats *via* the vasa vasorum or the perivascular lymph spaces. It is undecided whether the initial injury arises in the media or the adventitia. That the blood is capable of conveying the agent is borne out also by the experiments of von Haun (9) who

employed the blood derived from the vein of a living case for his animal inoculations.

In the reports of Adler (16), Hill (17), and Pearce (18) upon studies of the arterial system of normal rabbits no lesions suggestive of periarteritis nodosa are recorded. Lüpke (19) and Jaeger (20), however, describe a spontaneous disease in stags apparently identical with periarteritis nodosa of man. The possibility of the lower animal being the natural host for this virus is therefore to be considered.

CONCLUSIONS.

1. Periarteritis nodosa is a specific infectious disease which is transmissible to the rabbit.

2. The lesions induced in rabbits are identical with those occurring in man and consist of exudative and degenerative processes within the walls of the smaller arteries resulting in aneurysmal formations and thromboses.

3. The microorganism inducing the disease is capable of going through a Berkefeld N filter and is therefore to be classed with the group of so called filter passers.

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EXPLANATION OF PLATES.

PLATE 6.

FIG. 1. Photograph of the kidney showing multiple aneurysmal vessels of varying sizes with thrombi. These represent most of the lesions described as nodules. Where the section has not traversed the aneurysm the nodular aspect can be appreciated.

PLATE 7.

FIG. 2. Photomicrograph of an area in the kidney demonstrating lesions in the two arteries, one cut obliquely and the other transversely. The obliquely sectioned artery shows an inflammatory nodule involving the upper end of the artery and diffusing into the periadventitial tissues; extensive necrosis of a branch and also of the lower end of the artery proper is present. In the lower edge of the picture the artery cut transversely shows extensive necrosis down to the intima with no inflammatory reaction. Between these arteries is seen another artery not involved.

FIG. 3. Photomicrograph showing a higher magnification of the inflammatory nodule present at the upper end of the obliquely cut artery in Fig. 2. The involvement of the intima is shown.

PLATE 8.

FIGS. 4 and 5. Photographs of the nodules present on opposite surfaces of the lung of Rabbit E, inoculated with a filtrate derived from the organs of Rabbit B.

PLATE 9.

FIG. 6. Artery present in the lung of Rabbit C, second generation, inoculated with an organ emulsion of a first generation rabbit. This vessel demonstrates invasion of the periadventitial tissues and the adventitia and outer media with cells composed especially of the polymorphonuclear neutrophilic variety.

FIG. 7. Necrosis of arterial wall, with dilatation and thrombus formation present in the liver of Rabbit A. This rabbit was inoculated with the nodule emulsion obtained from the human case.

PLATE 10.

FIG. 8. The marked hypertrophy of the arterial walls described for several of the rabbits is herein demonstrated. This vessel also shows the presence in the adventitia of eosinophils and a few lymphoid and plasma cells. The section is from the lung of Rabbit B which was inoculated with the human nodular emulsion.

FIG. 9. Periarterial accumulation of lymphoid cells and proliferation of connective tissue cells present in the liver of the first generation Rabbit B. The artery is greatly injured and compressed.

PLATE 11.

FIG. 10. Section of a longitudinally cut artery in the lung of Rabbit E, inoculated with a filtrate obtained from a first generation rabbit. There is present on the upper surface a periarterial nodule formation.

FIG. 11. High magnification of the nodule present in Fig. 10, showing the cellular infiltration with destruction of the media and elastic membrane. The process extends to the intima and consists especially of cells of the polymorphonuclear neutrophilic variety.

PLATE 12.

FIG. 12. Section of the liver of Rabbit E, inoculated with a filtrate of the organs of a first generation rabbit, showing arterial necrosis and aneurysm formation with rupture and hemorrhagic extravasation into the liver tissue and beneath Glisson's capsule.

FIG. 13. Section of the liver of first generation Rabbit B, showing hemorrhagic extravasation into the surrounding tissue.

PLATE 13.

FIGS. 14 and 15. Low and high magnifications of an artery in the lung of Rabbit E. This animal had been inoculated with the filtrate of the organs of a first generation rabbit. The distinct periarterial nodular formation is prominent. The nodules are composed especially of lymphoid and plasma cells, with a few eosinophils and polymorphonuclear neutrophils. The involvement of the media by this process can be seen in Fig. 15.

PLATE 14.

FIGS. 16 and 17. Low and high magnifications of another artery in the lung of Rabbit E. The pathological processes are similar to those shown in Figs. 14 and 15, excepting that the distribution is more circumferential.



FIG. 1.

(Harris and Friedrichs: Periarthritis nodosa.)

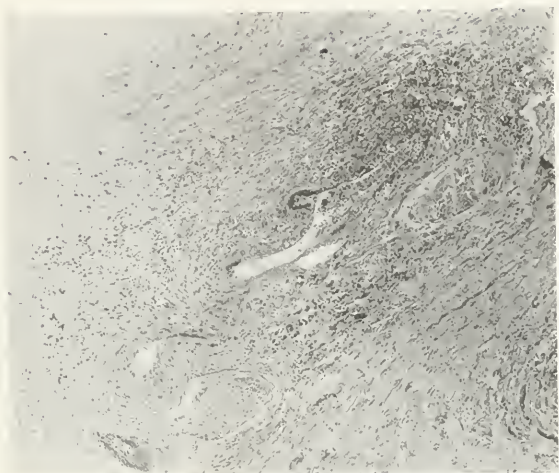


FIG. 2.

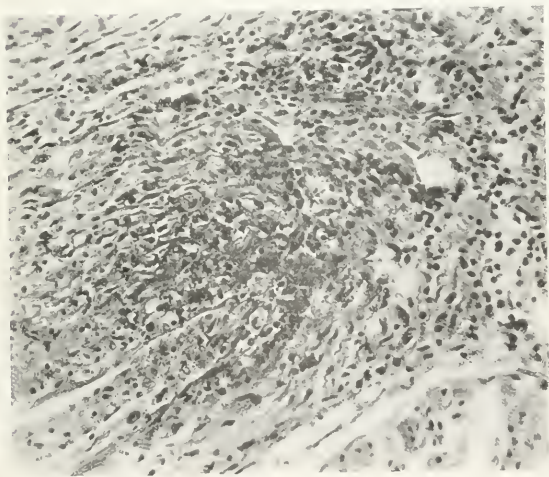


FIG. 3.

(Harris and Friedrichs: Periarteritis nodosa.)



FIG. 4.

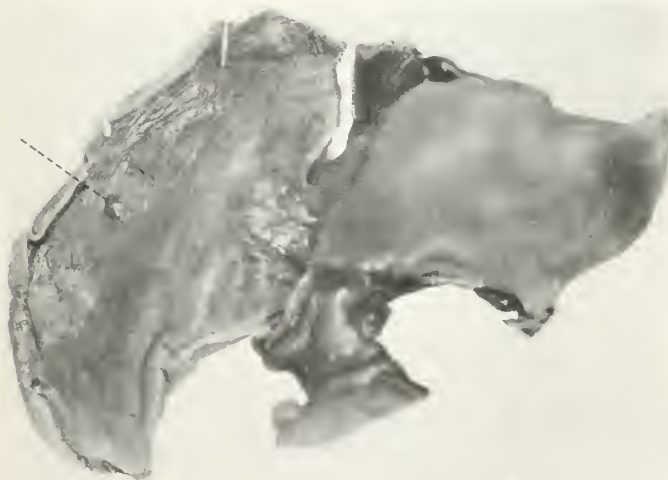


FIG. 5.

(Harris and Friedrichs: Periarthritis nodosa.)

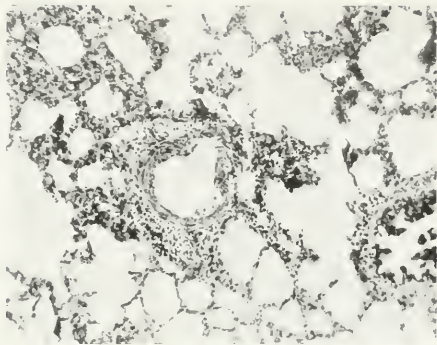


FIG. 6.

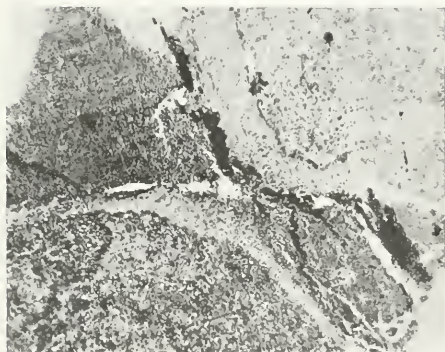


FIG. 7.

(Harris and Friedrichs: Periarthritis nodosa.)

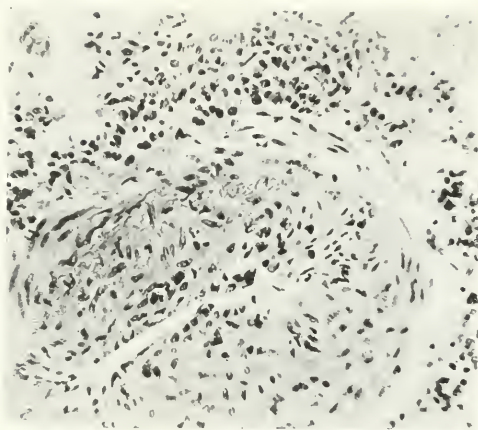


FIG. 8.

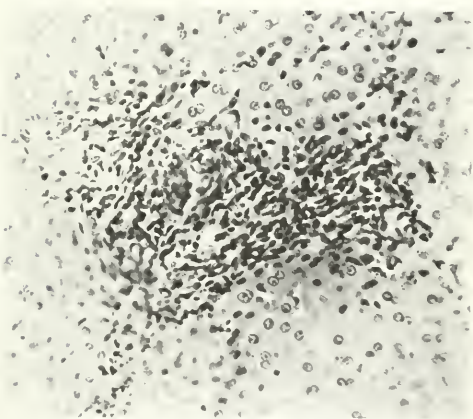


FIG. 9

(Harris and Friedrichs: Periarthritis nodosa.)

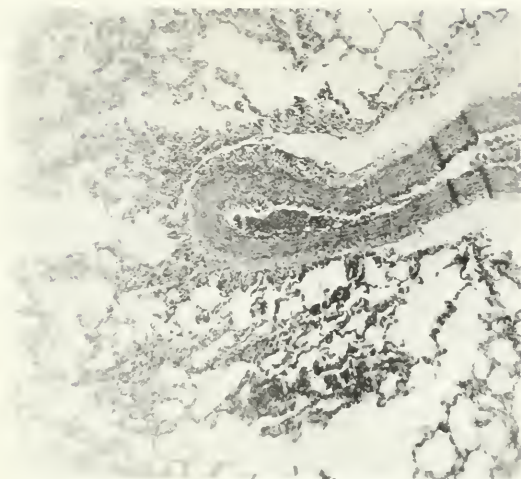


FIG. 10.

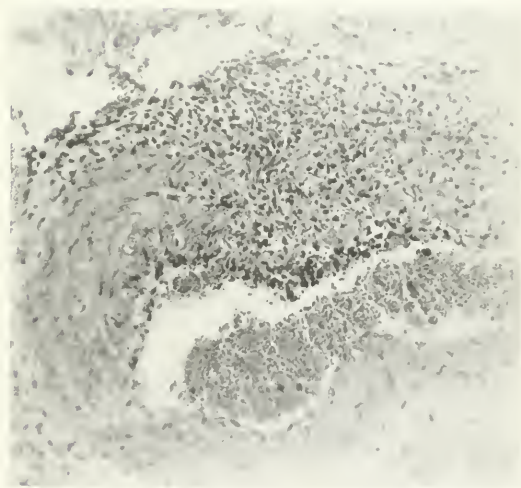


FIG. 11.

(Harris and Friedrichs: Periarteritis nodosa.)

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FIG. 12.

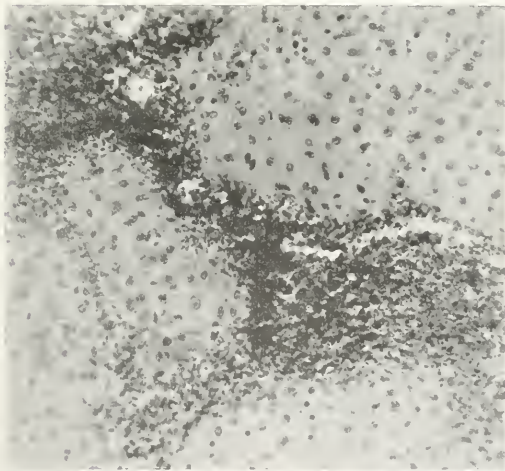


FIG. 13.

(Harris and Friedrichs: Periarthritis nodosa.)

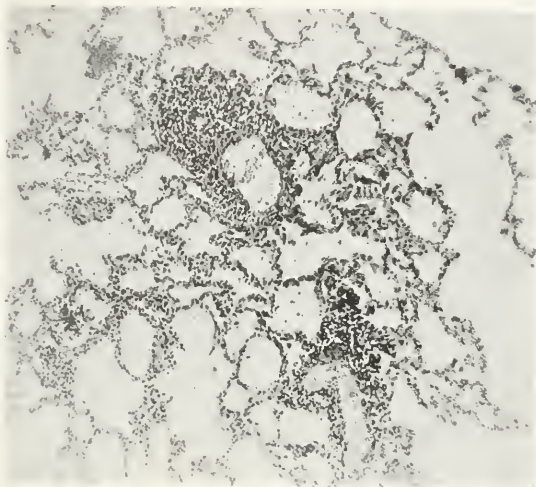


FIG. 14.

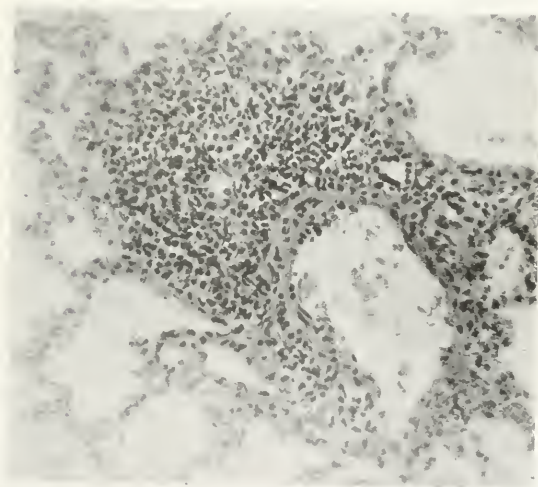


FIG. 15.

(Harris and Friedrichs: Periarteritis nodosa.)

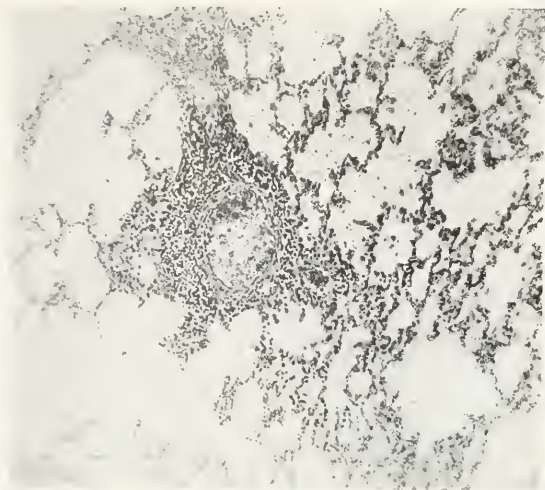


FIG. 16.

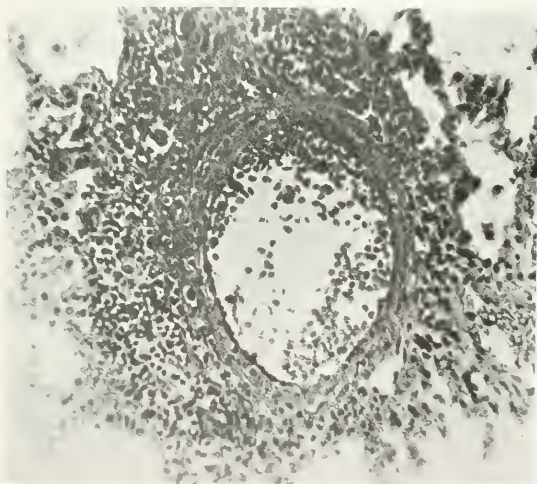


FIG. 17.

(Harris and Friedrichs: Periarthritis nodosa.)

STUDIES UPON EXPERIMENTAL MEASLES.

II. THE ENANTHEMATOUS, EXANTHEMATOUS, PYREXIAL, AND LEUCOCYTIC SYNDROME PRODUCED IN THE RABBIT BY INTRAVENOUS INOCULATION OF BLOOD FROM CASES OF HUMAN MEASLES.

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In a previous paper¹ we reported the successful propagation of the virus of measles in the guinea pig, the criteria of transmission being the constant occurrence of leucopenia, pyrexia, and nephritis following, after a definite incubation period, the intracardiac injection of blood from cases of human measles. For this host, however, no well defined rash, or exanthem, was noted. In the absence of any definite cutaneous reaction, the symptoms observed, although striking, did not permit of the conclusion that the identical clinical picture of the human infection had been reproduced. Therefore, the purpose of the present series of experiments was not only to determine whether the virus of measles could be propagated in the rabbit by direct transfer of infected blood from man, but also to ascertain whether in this animal the experimental infection would induce the characteristic syndrome of the human disease.

While we were engaged upon the experiments herein reported there appeared the observations of Nevin and Bittman² upon the effect of the virus of measles on the rabbit. These authors record the appearance of enanthem, exanthem, and pyrexia in a great proportion of rabbits infected by the intracirculatory injection of

¹ Duval, C. W., and D'Aunoy, R., Studies upon experimental measles. I. The effects of the virus of measles upon the guinea pig, *J. Exp. Med.*, 1922, xxxv, 257.

² Nevin, M., and Bittman, F. R., Experimental measles in rabbits and monkeys, *J. Infect. Dis.*, 1921, xxix, 429.

citrated blood from cases of human measles. Hardie³ also reports similar observations in which the rash appeared upon the shaved skin of rabbits on an average of 36 hours after the circulatory introduction of citrated blood from cases of measles.

EXPERIMENTAL.

Defibrinated human blood obtained from measles cases at the stage of temperature height was used for inoculation. The interval between collection of infected material and animal inoculation was never more than 1 hour, and during this intervening period the defibrinated blood was kept at a temperature of 37.5°C. Injections in rabbits were made into the marginal ear vein in a majority of cases, although in a few instances the intracirculatory introduction of infected material was accomplished by cardiopuncture.

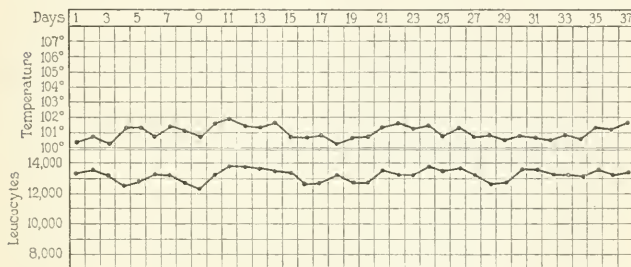
Experiment 1 (Preliminary).—The divergence of opinion regarding the normal variations in temperature and number of circulating leucocytes in rabbits made it necessary for us to determine these factors in order to establish a basis for the experiments. Twelve healthy animals were isolated, and their rectal temperatures and leucocytic counts determined daily for a period of 37 days. Text-fig. 1 represents the normal variations observed.

The results show that the average temperature of the rabbits was 101°F. In the experimental animal of this species we regarded as pyrexia only the reading about 103°F. The white elements averaged 13,000. Leucocytic counts below 9,000 are considered as evidence of leucopenia.

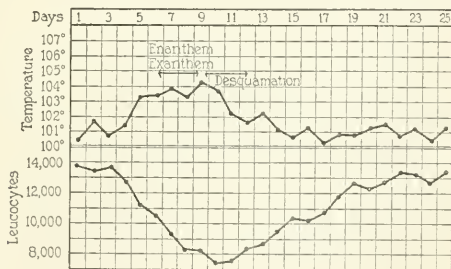
Experiment 2.—Here the purpose was to determine whether the virus of human measles could be directly carried from man to rabbit, and could induce in this host the clinical picture of the human infection. Blood from a typical case of human measles in the eruptive stage of the disease was employed as the inoculation material. Twenty rabbits comprised the number used in the experiment, sixteen being inoculated and four held as controls. The inoculated animals each received 2 cc. of human defibrinated virus blood injected into the marginal ear vein. Of the control animals two were isolated and injected intravenously with 3 cc. of normal human blood collected under aseptic conditions; the remaining two were not injected but left in the cage with the inoculated animals with the view of determining whether the infection

³ Hardie, M., Essais de transmission expérimentale de la rougeole au lapin. Constatacion d'un erythème sur la peau rasée, *Compt. rend. Soc. biol.*, 1921, lxxiv, 968.

could be transmitted through contact. Cultures made from the blood containing virus before inoculation remained sterile for ordinary bacteria. Of the sixteen rabbits receiving the blood from cases of measles, seven showed, after a period of approximately 5 days, reactions indicative of specific infection. There was a sharp



TEXT-FIG. 1. Composite temperature and leucocytic charts of twelve normal rabbits.



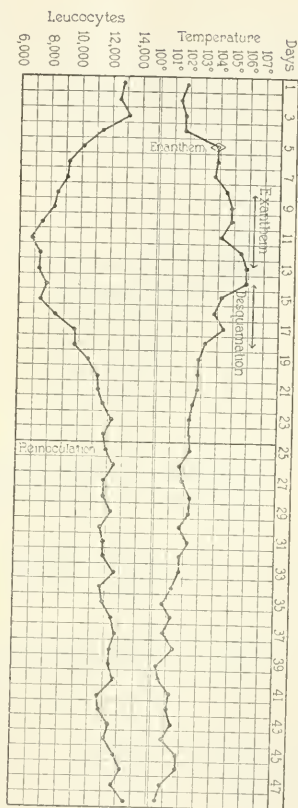
TEXT-FIG. 2. Typical reaction in the rabbit to blood containing the virus of measles.

rise in temperature of 2-3°F., beginning on or about the 5th day after inoculation in the majority of animals, with a coincident fall in the total number of circulating leucocytes. In some rabbits the rise in temperature did not appear before the 10th day. As a rule, the temperature remained high with slight variations for 2 to 4 days. During the pyrexial stage four animals developed an exanthem in the form of a dif-

fuse macular rash followed by the appearance of discrete papules over the neck and chest. At about the same time there were noted several sharply defined smaller erythematous patches on the buccal mucous membrane, resembling in the main the so called Koplik spots. The maculopapular eruption began to fade after 48 hours though it remained visible for 72 hours as a brownish discoloration. Branny or scaly desquamation invariably followed the rash. While only four of the seven reacting animals of this series presented a well defined rash, the leucocytic and temperature reactions in the remaining three were of sufficient significance to lead to the conclusion that the virus of human measles was capable of infecting the rabbit without giving rise to the cutaneous lesion. On the other hand, the appearance of an exanthem in the inoculated animal, coincident with the prominent and constant signs of pyrexia, leucopenia, coryza, etc., permits of the conclusion that the rabbit affords an experimental host in which the virus of measles produces the same clinical picture as observed in man. Text-fig. 2 graphically represents the typical reaction induced in the rabbit.

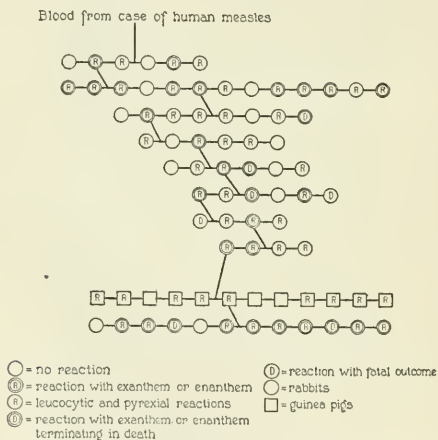
Experiment 3.—In order to determine the effects of reinoculation upon previously reacting animals, four rabbits of Experiment 2 which had shown typical exanthemata, leucocytic and pyrexial reactions were reinoculated, after complete recovery, with 2 cc. of blood from human and guinea pig measles cases. Two animals were used for the human virus, and two for the guinea pig virus, the blood containing the virus in both instances being obtained at the height of the reaction. In none of these animals were there noted any further changes in the temperature or cell count, nor the appearance of exanthemata. This would seem to prove that infection establishes an immunity (Text-fig. 3).

Experiment 4.—The purpose of this experiment was to continue the propagation of the virus by passage from rabbit to rabbit with the view also of increasing its virulence through repeated passage. Blood from animals reacting to the direct transfer of human measles virus was injected intravenously in 1 cc. quantities into twelve rabbits. In the animals of the second series the reaction was identical with that seen in the direct transfer animals. The proportion of reactions, however, was greater than with those of Experiment 2. Eleven of the rabbits reacted, nine of them showing exanthemata and enanthemata. After an incubation period of 3 to 5 days there occurred a rather abrupt rise in temperature of 1–4°F. and a coincident slight fall in the leucocytes. The temperature elevation continued usually for 24 to 36 hours in the majority of the animals; in the others it lasted with remission for 3 to 4 days. In the latter the leucocytes always showed the greatest decrease (well marked leucopenia). Furthermore, it may be said that the exanthem occurred in the animals with the more pronounced symptoms. In all, eight transfers from rabbit to rabbit were carried out, thus transmitting the virus through the eighth generation. In this experiment it is noteworthy that a number of rabbits died presumably as result of the virus, death occurring more frequently in animals of the later transfers. Such animals were visibly sick during the period of maximum reaction. Autopsies revealed grave nephritic changes, not unlike those reported by us for the measles-infected



TEXT-FIG. 3. Effects of reinoculation, with blood containing the virus of measles, of a rabbit which had reacted to a previous injection.

guinea pig. The kidneys were swollen, cloudy, and often infiltrated with hemorrhages. No noteworthy lesions were demonstrable in other internal organs, other than that common for toxemia. In no instance was pneumonia observed, nor were the ordinary pathogenic bacteria ever isolated from the heart's blood or internal organs of these fatal cases. This is of unusual interest because in fatal cases of human measles, pneumonia and septicemia almost invariably occupy the clinical field at death. While mortality is attributed in the human to secondary invaders, these seem to play no part in the experimental disease for rabbits and guinea pigs, the virus being capable *per se* of destroying the host. Text-fig. 4 expresses graphically the results of this experiment.



TEXT-FIG. 4. The continued reaction in the rabbit through successive generations, and the cross-reaction through the rabbit and guinea pig.

Experiment 5.—Since it was found that the rabbit as well as the guinea pig reacts in a characteristic manner to direct intravenous injections of human blood from cases of measles a series of experiments was carried out to determine what effects would be induced by transmission of the virus from rabbit to guinea pig and *vice versa*. Accordingly, one set of twelve guinea pigs received intracardially 1 cc. of fresh defibrinated rabbit blood obtained from an infected animal at the height of the reaction. Another set of twelve rabbits was inoculated intravenously with 1 cc. of fresh defibrinated blood secured from an infected guinea pig at the time of maximum pyrexia. Typical reactions were secured in 75 per cent of the cross-inoculated guinea pigs and in ten of the twelve rabbits.

DISCUSSION AND SUMMARY.

An active transmissible virus exists in the blood of measles patients during the eruptive stage of the disease. This virus produces in rabbits after intravenous injection a specific reaction analogous in all essential features to that of the human infection. Following a definite incubation period of from 2 to 5 days the animals infected show pyrexial, leucocytic, and cutaneous alterations. Fully 90 per cent of such inoculated rabbits react in a remarkable manner. The earliest constant symptom of the infection is a rise in temperature, which on the average occurs 4 days after inoculation and most probably marks the end of the incubation period. Concomitantly with this temperature rise there is a diminution in the total number of circulating leucocytes. This decrease in the number of white blood elements may be relative or may appear in the form of a well defined leucopenia. The most striking objective signs are the coryza, conjunctival injection, enanthemata, and exanthemata. The mucous membrane lesions are similar in their physical appearance to the so called Koplik spots seen in man. They occur on the buccal side of the oral cavity ranging in number from two to eight discrete hemorrhagic areas with paler centers. They appear as a rule coincidentally with the temperature rise or shortly thereafter. The exanthematous lesions though occurring only in about 40 per cent of the infected animals complete the clinical syndrome in this particular experimental host. The rash may appear as early as the 3rd and as late as the 7th day after inoculation. In its early stage it is of the macular variety, appearing as a diffuse eruption which later develops into a more papular type of lesion. At this time the cutaneous manifestations appear as slightly raised, flattened, purplish red, discrete areas in the skin of the face, neck, chest, and abdomen.

Repeated passage of the virus of measles through the rabbit seems to increase its virulence. A number of animals infected with such passage virus succumb in the fourth and subsequent generations, undoubtedly as the direct result of the action of the specific excitant, as in none of the animals was there cultural evidence of secondary intercurrent infection. In the animals dying presumably as a result of the specific virus grave nephritic changes were evident. It is a

noteworthy fact that the pneumonia so common in fatal cases of human measles was not evident in any of the experimental animals. We believe this to be of considerable significance, especially in elucidating the direct etiological factor of the fatal pneumonias so often present in human measles cases. Apparently such infections in man can be explained purely on the basis of the destruction of normal defense barriers by the specific excitant of the infectious disease, and the lack of host resistance to the ordinary pyogenic microorganism.

STUDIES UPON EXPERIMENTAL MEASLES.

III. THE SYMPTOM-COMPLEX IN THE GUINEA PIG AND RABBIT FOLLOWING THE INTRATRACHEAL AND INTRAVENOUS INJECTIONS OF FILTERED NASOPHARYNGEAL SECRETIONS FROM CASES OF HUMAN MEASLES.

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In previous communications^{1,2} it has been shown that guinea pigs and rabbits when injected intravenously with blood secured from cases of human measles at the height of the exanthem react specifically. This reaction consists in pyrexial changes, leucopenia, and in the great majority of animals enanthemata and exanthemata fairly characteristic of the human disease. In this study we desire to record the reaction produced in these animals as a result of the introduction into their circulatory and respiratory systems of filtered nasopharyngeal washings from cases of human measles.

LITERATURE.

That the nasopharyngeal secretions of patients suffering from measles contain the specific virus is at present unquestioned. Goldberger and Anderson³ by means of filtered and unfiltered buccal and pharyngeal secretions from cases of human measles injected into the respiratory tract of monkeys produced a symptom-complex which

¹ Duval, C. W., and D'Aunoy, R., Studies upon experimental measles. I. The effects of the virus of measles upon the guinea pig, *J. Exp. Med.*, 1922, xxxv, 257.

² Duval, C. W., and D'Aunoy, R., Studies upon experimental measles. II. The enanthematous, exanthematous, pyrexial, and leucocytic syndrome produced in the rabbit by intravenous inoculation of blood from cases of human measles, *J. Exp. Med.* 1922, xxxvi, 231.

³ Goldberger, J., and Anderson, J. F., The nature of the virus of measles, *J. Am. Med. Assn.*, 1911, lvii, 971.

they interpreted as mild manifestations of the disease. Blake and Trask⁴ working with unfiltered nasopharyngeal secretions introduced intratracheally into *Macacus rhesus* confirmed and extended these early observations. Grund⁵ by intratracheal injections of filtered and unfiltered nasopharyngeal secretions, obtained from the human case during the preeruptive and early eruptive stages of the disease, succeeded in producing a series of reactions in rabbits which were considered suggestive in a great proportion of the experiments as manifestations of the human disease.

EXPERIMENTAL.

The nasopharyngeal secretions used for these experiments were secured from cases of the human disease at the height of the cutaneous reaction. In obtaining the secretions sterile applicators tipped with cotton were introduced through West tubes into the nares and around the tonsillar surfaces. The soiled applicators were then washed in small portions of 0.85 per cent sterile sodium chloride solution previously kept at a temperature of 37°C. In a large number of instances as many as twenty infected applicators were obtained from a single individual, but the amount of saline solution used in washing those from one case was never more than 10 cc. In several cases the bacterial flora of the washings was studied. *Streptococcus hæmolyticus* and *non-hæmolyticus*, *Streptococcus viridans*, *Bacillus coli communis*, *Bacillus influenzae*, and *Staphylococcus aureus* were the only microorganisms cultivable by ordinary methods that were encountered. Filtration of washings was made through Berkfeld N filters, the filtrate in all instances showing no ordinary bacteria. From the time of securing washings to the time of animal inoculations not more than 1 hour elapsed, and during this time the washings were maintained at a temperature of between 37.5° and 39°C. Intratracheal injections were always made under ether anesthesia. The trachea of the experimental animal was exposed, it was then steadied, and the material was slowly injected into the upper and lower part of the respiratory tract through a needle inserted between the cartilaginous rings. As much as 10 cc. of fluid could thus be injected

⁴ Blake, F. G., and Trask, J. D., Jr., Studies on measles. I. Susceptibility of monkeys to the virus of measles, *J. Exp. Med.*, 1921, xxxiii, 385.

⁵ Grund, M., Susceptibility of rabbits to the virus of measles. Inoculations with nasopharyngeal material, *J. Infect. Dis.*, 1922, xxx, 86.

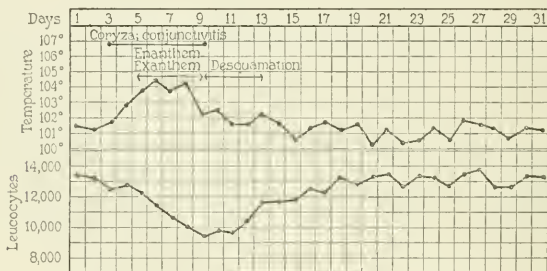
in a full grown rabbit with no subsequent discomfort to the animal. A single catgut suture was used to approximate the margin of the skin incision. In rabbits the material was injected into the marginal ear vein and in guinea pigs it was administered by cardiopuncture.

Experiment 1.—A number of rabbits (Table I) were injected directly into the trachea with 10 cc. of pooled filtered nasopharyngeal washings. At the same time a series of control animals was similarly injected with filtered washings secured from normal nasopharynges and from patients suffering with lobular pneumonia. Rectal temperature determinations, leucocytic counts, and observation for enanthemata and exanthemata were performed at stated intervals thereafter. All of the animals of the series receiving filtrates obtained from measles cases reacted similarly in as far as pyrexia and leucocytic depression were concerned. After an incubation period varying from 3 to 7 days there occurred an elevation of temperature amounting to as much as 3° in a number of cases.⁶ Coincident with the beginning of temperature rise, or shortly thereafter, enanthemata were noted in approximately 90 per cent of the animals. These appeared on the buccal and hard palate mucosæ as minute anemic areas surrounded by a pinkish to reddish zone. Exanthemata occurred in 60 per cent of the animals, appearing about the time of temperature height (5 to 9 days). The rash usually appeared first about the face, varying from a diffuse macular reaction to well marked papules. At the height of the cutaneous reaction well defined papules could be observed over the neck between the front quarters, and upon the abdominal wall of the animal. Invariably after the disappearance of the rash, branny or scaly desquamation took place. All the animals manifesting an exanthem appeared sick, and of these a number subsequently died (Table I). Con-junctival injection was always a prominent sign. In most of the animals no well marked leucopenia was noted, though a relative fall in the leucocytes always occurred, but this within wide limits. The control animals inoculated with filtered washings from other individuals failed to react in any manner. Text-fig. 1 expresses graphically the findings in a typical reacting animal of the series.

Experiment 2.—The susceptibility of the rabbit to the intracirculatory introduction of filtered nasopharyngeal washings was here tested. 5 cc. of filtrate obtained as above outlined were injected into the circulation of a number of full grown rabbits (Table I). These were observed over a period of 4 weeks, leucocytic and temperature curves being drawn for all animals. Of the series injected, 80 per cent showed unmistakable evidences of a reaction, which reaction we interpret as ascribable to the effects of the transmitted virus of measles. Again, in these series well marked leucopenia was not noted for all animals, but enanthem, exanthem, and temperature reactions were regularly encountered.

⁶ As in the previous study we consider only temperatures of 102°F. or over as pyrexia.

Experiment 3.—Intratracheal injections of 2 cc. portions of filtered washings were made in a number of guinea pigs (Table I). Of the animals thus injected, 75 per cent reacted in a manner identical to that previously reported by us as characteristic when blood from cases of human measles was injected into the guinea pig. Regularly in the reacting animals 9 to 14 days after injection, the temperature rose with a coincident fall of the circulating leucocytes. 10 per cent of the injected animals succumbed with no gross lesions discernible at necropsy, excepting an acute hemorrhagic nephritis. Animals dying failed to show in their blood stream or in their organs by ordinary cultural methods any microorganisms which could be considered responsible for death. Typical enanthemata and exanthemata were not observed in any of the guinea pigs of the series; however, in a number a few discrete hemorrhagic areas were noted on the buccal mucous membranes before the appearance



TEXT-FIG. 1. Reaction in the rabbit following intratracheal injection of filtered nasopharyngeal material from cases of human measles.

of the temperature rise, but as these were not grossly characteristic as compared to the Koplik spots seen in man or the lesions noted in the rabbit after experimental transmission of the virus of measles, they are not stressed in the present report. Exanthemata are either evanescent, difficult of recognition, or do not occur in the guinea pig, as we failed to note such cutaneous manifestations in any of the animals. Attempts at removing the hair from the abdomen of normal guinea pigs, either by shaving, clipping, or cutting with scissors, caused confusing erythematous lesions of from 2 to 4 days duration.

Experiment 4.—2 cc. portions of filtered washings were introduced into the circulation of several guinea pigs of average weight of 250 gm. 90 per cent of the animals thus injected reacted typically after incubation periods varying from 7 to 11 days (Table I). Well marked leucopenia was observed in all the reacting animals of the series. Subcultures from blood obtained from the reacting animals of this series

when injected into rabbits gave rise after definite incubation periods to typical reactions characterized by temperature changes, exanthemata, and enanthemata.

Experiment 5.—Five guinea pigs and five rabbits that had reacted characteristically after the intratracheal injection of filtered washings and had completely recovered, were given intracirculatory injections of defibrinated blood secured from cases of human measles at the height of eruption. None of these animals reacted in any manner. This is in keeping with previous observations upon the immunity in the guinea pig conferred by the intracirculatory injection of blood from cases of human measles.

TABLE I.

Reaction in Rabbits and Guinea Pigs Following Inoculation with Nasopharyngeal Material from Cases of Human Measles.

Experimental animal.	No. of animals inoculated.	Method of inoculation.	No. of animals reacting.						Mortality.
			Enanthen.	Exanthen.	Conjunctivitis and coryza.	Desquamation.	Leucopenia.	Pyrexia.	
Rabbit.	12	Intratracheal.	10	7	10	7	Marked, 4. Relative, 5.	11	2
"	10	Intracirculatory.	8	8	8	8	Marked, 2. Relative, 6.	9	1
Guinea pig.	12	Intratracheal.	3	0	0	0	Marked, 9.	11	2
" "	10	Intracirculatory.	4	0	0	0	" 9.	10	3

DISCUSSION.

As a result of the experiments above recorded it seems conclusively established that the intratracheal and intracirculatory introductions in guinea pigs and rabbits of filtered nasopharyngeal secretions from cases of human measles occasion a definite and constant reaction. This reaction is characterized by a complex of objective and subjective signs which very closely resemble the manifestations in man of the acute infectious disease measles. In the guinea pig the incubation period following injection ranges between 9 and 14 days, approximately the same length of time previously noted by us following the intracirculatory introduction in this animal of defibrinated blood from cases of measles. Leucopenia is always a constant change, appearing about the time of temperature rise or shortly before, and lasting throughout the period of pyrexia. Enanthemata, never quite characteristic grossly as compared to Koplik's spots in

man, are fairly constant, appearing at the period of pretemperature or early temperature rise. Histologically, however, these enanthematous lesions present the identical changes described by Mallory and Medlar⁷ in human Koplik spots. Exanthemata were never definitely noted. We believe that they are difficult to elicit in the guinea pig. We were often confused by the peculiar erythema, appearing in normal guinea pigs after the removal, by shaving or cutting, of hair. This non-specific macular rash persisted in a number of instances for from 3 to 4 days, being followed by a light desquamation. Conjunctival irritation is always prominent in reacting animals. Repeated aerobic and anaerobic cultures from the conjunctival sacs of such animals failed to reveal organisms deserving any special mention.

In the rabbit, reaction always manifests itself early, on an average in 4 days, the extreme limits noted in our series being 2 and 7 days. Conjunctival injection and enanthemata are generally the early evidences of the symptom-complex. The buccal mucosal lesions appear first as anemic areas surrounded by an injected areola, and generally develop into distinct papules. As many as eight such papules can be noted in some of the animals. At the time of enanthem appearance or shortly thereafter a sharp rise of temperature takes place, amounting to as much as 4° in most cases. With the appearance of pyrexia, the cutaneous reaction manifests itself. As a rule, the exanthem appears first about the face in the form of a diffuse macular eruption. Soon afterward a similar rash appears on the neck, between the front quarters, and on the abdominal wall. Typical papules now develop, which are present for from 1 to 3 days. With the onset of papules there is usually a fall in temperature, with a gradual return to normal. Desquamation, usually branny, sometimes scaly, appears regularly. There is always a relative drop in the circulating leucocytes, at the expense of the neutrophilic element, without, however, the very sharp decline noted in the guinea pig.

The regularity with which it has been possible to produce this symptom-complex in guinea pigs and rabbits following the injection of filtered nasopharyngeal washings from cases of human measles and the similarity of the symptom-complex to the manifestations

⁷ Mallory, F. B., and Medlar, E. M., The skin lesion in measles, *J. Med. Research*, 1919-20, xli, 327.

of measles both in man and in the guinea pig and rabbit following the intracirculatory injection of blood from cases of human measles, lead us to agree with the conclusion that there is present in the nasopharyngeal secretion of measles cases as a causal agent of the disease a filter-passing virus.

We believe that experimental measles, at least in the rabbit and guinea pig, is primarily an infection of the blood, and that the mucous membrane lesions are secondary. It seems necessary that the virus should enter and propagate in the blood stream before any manifestations of the infection present themselves. The coryza, conjunctivitis, and other early signs are not to be regarded as indications of a primary mucous membrane localization of the specific excitant, but are the earliest outward evidences of an already established blood infection. The course of the experimental infection in these animals varies but little whether the infective material is nasopharyngeal secretions or blood, and whether the inoculating route is intravenous or intratracheal. This is not surprising when we consider that practically instantaneous absorption⁸ of intratracheally deposited material takes place, naturally with rapid entrance into the circulation. If the localization of the specific incitant took place in the mucous membranes by the selective action of this incitant, it would seem plausible to expect an earlier manifestation of both objective and subjective signs following the direct infection of mucosal surfaces than following infection by the intracirculatory route.

CONCLUSIONS.

1. There is present as a causal agent in the nasopharyngeal secretions of measles cases a filter-passing virus.

2. The rabbit and the guinea pig react specifically to the intratracheal and intracirculatory injections of filtered nasopharyngeal secretions secured from cases of human measles.

3. Enanthem, exanthem, and pyrexial disturbances characterize this specific reaction in the rabbit; in the guinea pig the reaction manifests itself by pyrexia, marked leucopenia, and grave nephritis in the fatal cases.

⁸ D'Aunoy, R., Antibody production after intratracheal injection of antigen, *J. Infect. Dis.*, 1922, xxx, 347.

CHANGES IN THE NUMBER OF SMALL LYMPHOCYTES OF THE BLOOD FOLLOWING LIGATION OF THE THORACIC DUCT.

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INTRODUCTION.

The important work of Murphy and his collaborators has added great interest to the study of the physiology of the lymphocyte. These investigators have shown particularly well the rôle that the lymphocyte plays in the resistance and susceptibility of mice to cancer grafts. Previously the lymphocyte was studied considerably in regard to the extent of its appearance in the thoracic duct under ordinary conditions or its appearance in the blood stream following splenectomy (Biedl and von Decastello), after extirpation of groups of important glands (Ehrlich and Reinbach), after the administration of pilocarpine (Rous), and following ligation of the thoracic duct (Davis and Carlson; Bunting and Huston).

During the progress of work on the ligation of the thoracic duct recently reported (Lee), the opportunity offered itself for studying the effect of the ligation of that vessel on the number of small lymphocytes in the blood.¹

Materials and Methods.

Young adult male cats were used for all of the experimental work. From blood which was procured from the ear of the animal, a white blood count and a smear were obtained. Wright's stain was applied to the smear and a differential count of 300 cells or more was made. Two control counts were made; one was obtained on the day preced-

¹ All operations were performed under ether anesthesia.

ing operation, and the other was taken 24 hours later, on the morning of the day of operation. The care of the animal before, during, and after the operation was the same as reported previously; likewise, the operation, which was essentially an intrathoracic ligation of the thoracic duct and the periaortic lymphatic plexus, was similar. 5 hours after operation the blood was examined again. On the following day counts were made in the morning and afternoon, while from then on the examinations were made in the morning of the next 7 successive days, and after that on alternate days. At the end of about 3 weeks the lymphocytes were again up to the preoperative level, and the animal was then sacrificed after the mesenteric lymphatics had been injected with Berlin blue. Blocks of tissue from the various organs were fixed in formaldehyde for further study, and a dissection of the collateral circulation of the thoracic duct was made.

Since the data from the entire series of five animals are about the same, it is only necessary to report the findings in two animals in order to illustrate the changes in the number of small lymphocytes in the circulating blood.

In the differential counts here reported the white blood cells have been classified under the following headings: polymorphonuclear neutrophils, eosinophils, basophils, small lymphocytes, large lymphocytes, large mononuclears, and transitionals. The polymorphonuclear neutrophils need no discussion. The eosinophils were not constant with respect to their granulation; the size of the granules was constant for any particular cell, though it varied for the different cells. Busch and Van Bergen have classified eosinophils into three groups according as the granulation was fine, medium, or coarse; yet it is obvious that such a classification is difficult. In all the smears that were examined a typical basophil was not seen. Very infrequently a cell was seen which had an irregularly shaped nucleus with a slightly granular purplish red cytoplasm; but the granulation was not typical of a basophil.

The small lymphocyte was taken to be a cell smaller than the polymorphonuclear neutrophil, round, with a round nucleus which was sometimes slightly indented. The nucleus was clearly outlined, and occupied almost the entire cell; its chromatin was dense, often aggregated, and deeply purple-stained. The cytoplasm was scant,

slightly blue-stained as a rule, with a small colorless rim around the nucleus, and it frequently contained a few fine azurophil granules.

The large lymphocyte differed from the small lymphocyte mainly in size, since the staining was essentially the same; there is a possibility, then, of considering these two cells as belonging to the same class. The nucleus of the large lymphocyte was larger than that of the small lymphocyte, the cytoplasm surrounded the nucleus definitely, and the azurophilic granules were larger and more numerous.

The large mononuclear was the largest blood cell observed in the cat; it was often several times as large as the polymorphonuclear neutrophil. The nucleus was large, often oblong, sometimes slightly indented; the chromatin was loose and skein-like and did not stain heavily. The cytoplasm was abundant, usually slightly granular or mottled, and contained, as a rule, numerous fine azurophil granules. It was often difficult to distinguish between this cell and the large lymphocytes, and undoubtedly the tables herewith submitted suffer because of this difficulty.

The transitional cell was considered to be similar to the large mononuclear and different mainly in that it had a markedly indented nucleus.

The recent work of Sabin has shown how vitally stainable granules may serve as a specific criterion for the differentiation of the three strains of the white blood cells of the chick; and there is reason to believe that this method may be applied generally to all blood examinations and can thus supplement the routine differential blood counts. Simpson has described how the vital dyes may serve to differentiate large mononuclear cells from transitional cells. Thorne and Evans also have employed vital dyes in showing the very low monocyte count of lymph in rabbits.

EXPERIMENTAL DATA.

With these standards for the identification of the cells in the differential counts made with Wright's stain, the experimental observations were carried out as described. The general technical procedures are indicated in the protocol of Cat 1, which is given below in condensed form.

Cat 1.—May 9, 1921. Young adult male cat, black; in stock since May 6, 1921. 10 a.m. Count made.

May 10. Food withheld for the last 24 hours. Weight 3,135 gm. 10 a.m. Count made. 11 a.m. Ligation of thoracic duct according to method already described. 5 p.m. Count made.

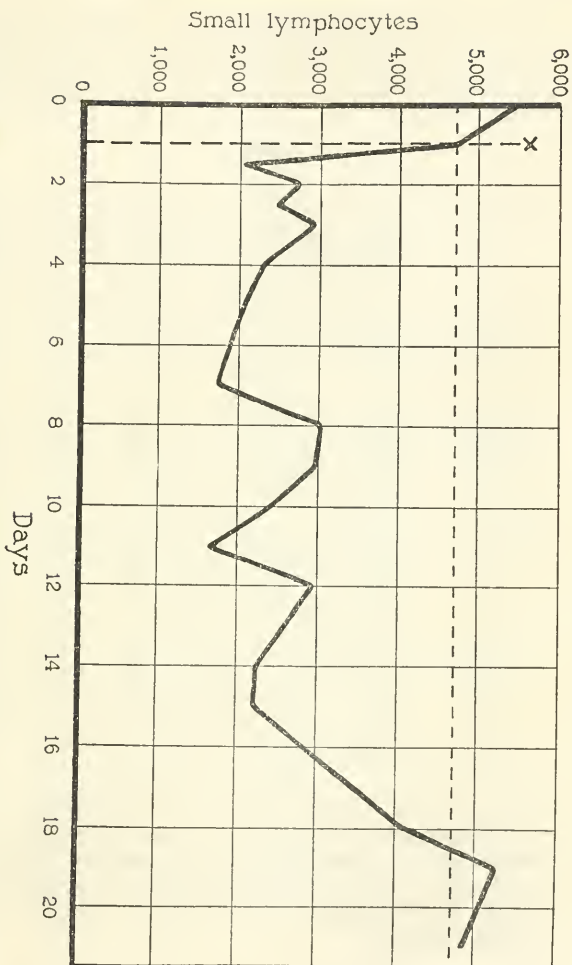
TABLE I.

Cat 1.

Date.	Total white blood count.	Poly-morpho-nuclear neutrophils.	Eosino-phils.	Small lympho-cytes.	Large lympho-cytes.	Large mono-nu-clears.	Transi-tionals.	Absolute No. of small lympho-cytes.
1921		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
May 9	15,720	51.3	11.0	35.0	1.0	1.3	0.3	5,502
" 10, a.m.	13,880	50.3	11.7	34.0	1.3	1.7	1.0	4,689
Operation.								
May 10, p.m.	30,600	88.3	0.7	6.7	2.7	1.0	0.7	2,050
" 11, a.m.	23,640	83.3	0.7	11.7	2.0	1.7	0.7	2,765
" 11, p.m.	22,800	84.0	1.0	10.7	2.0	1.3	1.0	2,439
" 12	19,900	75.7	4.3	14.7	2.7	1.7	1.0	2,925
" 13	14,280	70.7	10.7	16.3	1.3	0.7	0.3	2,327
" 14	13,880	71.3	11.7	15.0	0.7	1.0	0.3	2,080
" 16	13,760	73.7	9.0	12.3	4.0	0.7	0.3	1,692
" 17	17,400	70.7	4.3	17.3	3.7	3.3	0.7	3,010
" 18	13,800	66.7	6.0	21.3	3.3	1.7	1.0	2,939
" 19	13,600	68.7	6.3	17.7	3.0	3.3	1.0	2,407
" 20	10,560	71.7	7.3	15.7	1.0	3.3	1.0	1,657
" 21	12,000	65.3	7.0	24.7	1.0	1.3	0.7	2,964
" 23	12,520	70.7	8.7	18.3	0.7	1.0	0.7	2,291
" 24	10,800	71.3	6.3	20.3	1.0	0.7	0.3	2,192
" 27	16,640	60.7	10.0	24.7	3.3	1.0	0.3	4,109
" 28	18,000	56.3	8.7	29.0	2.7	2.7	0.7	5,220
" 30	17,440	60.7	9.7	27.3	1.3	0.7	0.3	4,861

Cat made uneventful recovery; the incision healed perfectly. Counts made are listed in Table I, and shown in Text-fig. 1.

May 30. Weight 3,420 gm. Since counts showed that the number of small lymphocytes in the circulating blood had reached the same level as before operation, the animal was sacrificed; the mesenteric lymphatic vessels were injected as described, and it was found that a lymphatovenous communication had established itself between the thoracic duct and the ninth left intercostal vein. The specimen was fixed in formaldehyde.



In all the figures the horizontal broken line indicates the level at the time of operation.

TEXT-FIG. 1. Curve to show the absolute number of small lymphocytes per cubic millimeter of blood in Cat 1 in which the thoracic duct was ligated at X. On the 19th day the number of small lymphocytes had again reached its preligation level.

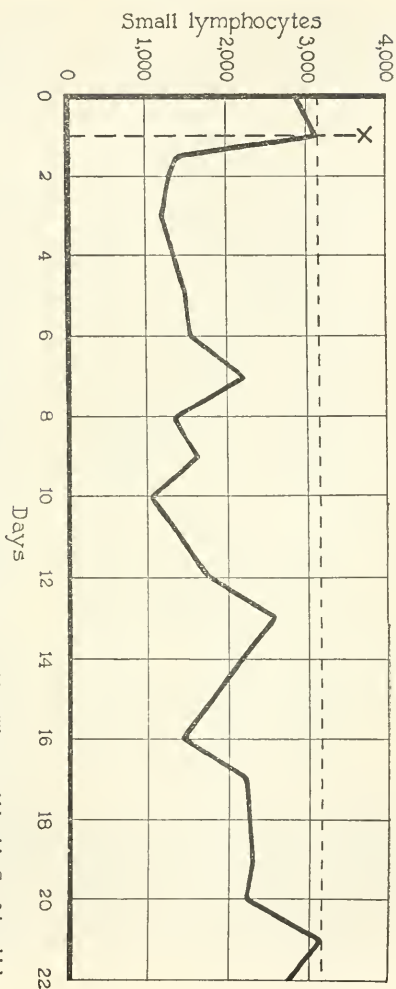
In this animal (No. 1) the number of small lymphocytes decreased 56 per cent immediately following the ligation of the thoracic duct. This reduction in the number of lymphocytes in the circulating blood endured until the preligation level was attained on the 21st day.

TABLE II.

Cat 2.

Date.	Total white blood count.	Poly-morpho-nuclear neutrophils.	Eosino-phils.	Small lympho-cytes.	Large lympho-cytes.	Large mono-nu-clears.	Transi-tionals.	Absolute No. of small lympho-cytes.
<i>1921</i>		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
May 25	13,400	71.0	4.7	21.0	1.3	1.0	1.0	2,814
" 26, a.m.	13,080	69.3	4.7	24.0	1.0	0.7	0.3	3,139
<i>Operation.</i>								
May 26, p.m.	38,160	94.0	0.8	3.6	0.6	0.8	0.2	1,373
" 27, a.m.	19,240	90.7	0.3	6.7	1.3	0.7	0.3	1,288
" 28	20,760	92.0	1.0	5.7	2.0	1.0	0.3	1,183
" 30	26,360	90.0	0.7	5.7	1.3	1.0	1.3	1,482
" 31	22,320	86.3	3.0	7.0	1.3	2.0	0.3	1,562
June 1	20,600	79.7	5.0	11.0	2.7	1.3	0.3	2,266
" 2	16,800	84.3	4.0	7.7	2.7	1.0	0.3	1,293
" 3	18,000	86.3	2.3	9.0	2.0	0.3	0	1,620
" 4	21,280	90.0	2.0	5.0	1.3	1.3	0.3	1,064
" 6	20,840	82.7	5.3	8.3	1.7	1.0	1.0	1,729
" 7	19,400	77.0	6.0	13.3	2.3	1.0	0.3	2,580
" 8	19,120	80.7	4.7	11.3	1.3	1.0	1.0	2,160
" 10	17,000	83.7	5.0	8.3	1.0	1.3	0.7	1,411
" 11	16,800	77.0	5.7	13.3	1.3	1.3	1.3	2,234
" 12	11,480	72.3	3.3	20.0	1.7	1.7	1.0	2,296
" 14	12,960	72.7	6.3	17.3	2.0	1.3	0.3	2,242
" 15	14,640	69.0	3.7	21.3	4.3	1.3	0.3	3,118
" 16	18,400	80.3	2.0	14.7	1.3	1.3	0.3	2,704

At this time an anatomical pathway capable of affording ingress of cells of the thoracic duct lymph into the systemic blood stream was demonstrated as a lymphatovenous communication between the thoracic duct and the ninth intercostal vein. The experiment was free from complications; absence of infection served to render the blood counts dependable.



TEXT-FIG. 2. Curve to show the absolute number of small lymphocytes per cubic millimeter of blood in Cat 2 in which the thoracic duct was ligated at X. On the 21st day the number of small lymphocytes had reached its preligation level.

A somewhat similar condition was shown by Cat 2, which weighed 3,030 gm. before operation, and 3,000 gm. when sacrificed. In this case, which likewise was free from concurrent infection, the lymphato-venous connection was between the thoracic duct and the azygos

TABLE III.

Cat 3.

Date.	Total white blood count.	Poly-morpho-nuclear neutrophils.	Eosino-phils.	Small lympho-cytes.	Large lympho-cytes.	Large mono-nu-clears.	Transi-tionals.	Absolute No. of small lympho-cytes.
<i>1921</i>		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
Apr. 25	15,920	78.0	3.3	12.7	2.7	1.3	2.0	2,021
" 26, a.m.	14,840	71.0	6.0	18.3	3.0	1.0	0.7	2,715

Operation.

Apr. 26, p.m.	18,040	88.0	0.3	7.7	1.3	2.0	0.7	1,389
" 27, a.m.	15,680	71.3	3.7	20.7	2.3	1.3	0.7	3,245
" 28	14,120	70.0	2.0	20.0	5.3	1.7	1.0	2,824
" 29	15,920	73.7	2.3	21.0	1.3	0.7	1.0	3,343
" 30	12,320	59.3	3.3	34.0	1.7	0.7	1.0	4,188
May 2	12,960	63.3	1.7	32.7	1.3	0.7	0.3	4,237

Cat 4.

Apr. 25	8,480	54.7	3.0	38.3	2.0	1.3	0.7	3,247
" 26, a.m.	8,360	61.0	3.0	32.7	2.0	1.0	0.3	2,733

Operation.

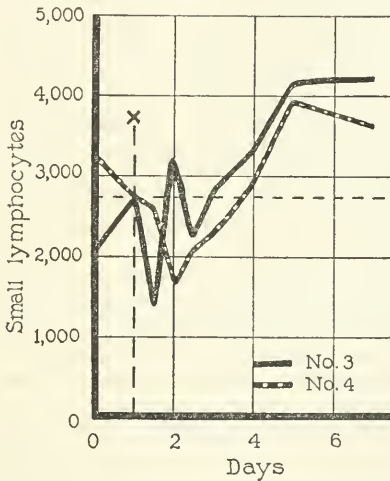
Apr. 26, p.m.	17,640	79.7	0.7	14.7	3.0	1.3	0.7	2,592
" 27, a.m.	9,680	77.3	1.7	16.7	1.7	2.0	0.7	1,616
" 27, p.m.	9,840	75.7	1.3	21.0	1.0	0.7	0.3	2,066
" 28	7,560	61.7	5.0	30.0	1.7	1.3	0.3	2,268
" 29	9,200	58.3	7.3	32.0	1.3	0.7	0.3	2,944
" 30	11,920	57.3	7.0	33.3	1.0	1.0	0.3	3,969
May 2	11,280	58.3	5.0	32.0	1.7	1.7	1.3	3,609

vein at the ninth interspace. The number of lymphocytes in the circulating blood also decreased by 56 per cent immediately following ligation, but gradually returned to the preligation level on the 19th day. Table II and Text-fig. 2 show the counts in this animal.

In order to check up the findings reported above, two control experiments were made. These animals were treated identically like

the ones above mentioned, with the exception that instead of performing the actual ligation in the chest, the operative procedure was limited to a slight dissection of the anterior periaortic tissue. The animals made a good recovery, and their blood counts are listed in Table III, and demonstrated in Text-fig. 3.

From the data herewith submitted it is seen that the intrathoracic ligation of the thoracic duct caused an immediate fall of about 56



TEXT-FIG. 3. Curve to show the absolute number of small lymphocytes per cubic millimeter of blood in Cats 3 and 4 which were used as controls. On the 4th day the number of small lymphocytes had already reached its preoperative level.

per cent in the number of small lymphocytes in the circulating blood, and that the number gradually returned to the preoperative level about 3 weeks after ligation (Text-figs. 1 and 2). In the one case the lymphatovenous connection was with the ninth left intercostal vein; and in the other the connection was with the azygos vein directly. It seems then that at the end of 21 days these lymphatovenous connec-

tions were of sufficient size to perform the functions previously exercised by the thoracic duct. Whether one can use the number of small lymphocytes in the blood as an absolute criterion for the degree of establishment of the collateral circulation is still questionable; nevertheless, the counting of the small lymphocytes is one of the more obvious and accessible methods for attacking this problem.

It will be noted that the decrease in the number of lymphocytes in the blood as shown by Tables I and II was invariably around 56 per cent; in the entire series this decrease has been between 54 and 64 per cent. This figure is somewhat lower than that of the only experiment in the series in which the ligation of both thoracic ducts alone was performed (No. 10, Biedl and von Decastello) in which a decrease of 79 per cent was obtained. In this case the thoracic duct on the left side, as well as all visible lymphatic vessels in the right side of the neck, was ligated. It is obvious that such a procedure shut off more lymphatic vessels than a simple ligation of the thoracic duct in the chest, and consequently one would expect a greater decrease in the number of lymphocytes in the circulating blood. For if the thoracic duct is obstructed in the chest, then the lymph of the upper extremities, head, neck, and upper part of the thorax, may still enter the blood stream through normal channels and thus furnish a considerable number of lymphocytes to the blood stream.

The control experiments showed that although there was also a decrease in the number of small lymphocytes when the duct had not been tied, yet this decrease was only temporary, for the preoperative level was exceeded on the 2nd or 3rd day (Text-fig. 3). Other cats which simply received an ether anesthetic for a half hour showed a temporary fall in the number of small lymphocytes.

DISCUSSION.

Many investigators have studied the thoracic duct with respect to the number and kind of cells for which it forms the pathway to the blood stream. Winternitz in 1895 concluded that the interruption of the lymph flow into the blood *via* the thoracic duct does not prevent leucocytosis. In 1901, Biedl and von Decastello described changes in the blood picture of dogs in which either a fistula of the duct was made with or without splenectomy, or in which the duct was tied under aseptic technique. They found that the mononuclears, including the small

lymphocytes, decreased in the blood from 62 to 79 per cent in absolute numbers. They recorded only two experiments, Nos. 9 and 10, in which they ligated the duct and studied the blood. In Experiment 9 ligation of the duct produced practically no change in number of mononuclears in the blood; this condition was accounted for by the demonstration of a branch of the thoracic duct below the point of ligation which connected with veins on the right side of the neck. In the other experiment, No. 10, the thoracic duct as well as all lymphatics on both sides of the neck were ligated, and it was found that the number of mononuclears in the blood diminished 79 per cent on the day following ligation, but on the 4th day after ligation the decrease was only 29 per cent. Blood counts were made until 1 week after operation. No attempt was made to determine whether the mononuclear cells in the blood would come back to the preligation level. Furthermore, the animal in Experiment 10 had an infection of the operative wounds; though the infection cleared up, nevertheless the inflammatory process undoubtedly brought in an unwelcomed factor which must have influenced the blood examination. The authors concluded that ligation of the thoracic duct or production of a fistula of the same did not entirely prevent the entrance of mononuclears into the blood stream; nor did injections of a colored solution serve to demonstrate connections between the lymph and blood systems. However, more recent work has shown that collateral paths may become established after the thoracic duct has been ligated.

In 1904 Selinoff reported experiments on dogs in which he cannulated the thoracic duct under aseptic precautions and studied the resultant change in the number of cells in the blood stream. He divided the white cells into three groups, young, adult, and old. This division corresponds essentially to lymphocytes, monocytes, and polymorphonuclears, respectively. In one experiment, No. 18, the lymphocytes dropped from 1,600 before operation to 20 on the 14th day after operation. However, the remarkable decrease in lymphocytes was attended also by a decided increase in the number of polymorphonuclears; thus the animal in the experiment cited above had on the 14th day polymorphonuclears present in the blood to the extent of 99.7 per cent. Not only did this experiment show a high polymorphonuclear content, but other experiments which were extended only to the 5th day after operation, such as No. 12, showed polymorphonuclears in the blood to the extent of 98.0 per cent. Obviously infection was present in all of the operative wounds of these experimental animals because of the thoracic duct fistula. It would seem then that in these cases the infection was characterized not only by a great increase in the number of polymorphonuclear leucocytes but also by an astonishing decrease in the number of lymphocytes in the circulating blood.

Also in 1904 Banti, in reporting the work done in his laboratory by Crescenzi, stated that after splenectomy and subsequent establishment of a thoracic duct fistula in dogs the lymphocytes of the blood decreased from one-half to ten-elevenths, but returned to normal or above normal at the end of 4 days, at which

time, it was claimed, there did not exist any accessory lymphatic channels between the thoracic duct and the subclavian vein. He was at a loss to account for the manner in which the lymphocytes reached the blood stream and considered as a probability the entrance of lymphocytes directly into the blood stream while still in lymphatic organs.

In 1908 Rous, using an improved technique, in the collection of samples from thoracic duct fistulæ showed that in dogs the thoracic duct furnished the circulating blood more lymphocytes than had been usually supposed. Also, he stated that muscular activity and certain lymphagogues increased the lymphocyte output.

Davis and Carlson in 1909, in studying the leucocytes in the neck lymph, thoracic lymph, and blood of normal dogs, also noticed a decrease in the number of the mononuclear elements of the blood following ligation of the thoracic duct and neck lymphatics. Unfortunately the observations were carried out for only 48 hours following operation and the subsequent course of the lymphocyte content of the blood was not followed; likewise it is uncertain to what extent the operation did prevent the entrance of lymph into the blood stream, since the lymphatics were not subsequently injected.

Bunting and Huston were also interested in the fate of the lymphocyte,—a question which Davis and Carlson had previously considered. Bunting and Huston splenectomized rabbits and found that the number of lymphocytes in the circulating blood increased. About a week following splenectomy the left thoracic duct as well as the neck lymphatics on both sides were ligated, and it was found that the number of lymphocytes in the circulating blood decreased. They also observed lymphatics going from the left thoracic duct through the thymus to the right thoracic duct, and concluded that the lymphocyte count was restored through such channels; however, they did not keep their animals long enough to note the return of the lymphocytes to their preoperative level. The authors advanced the hypothesis that the lymphocytes may migrate for instance into the mucous membrane of the intestine and from there into the lumen of the intestine, since more lymphocytes enter the blood stream *via* the thoracic duct in 24 hours than are present in the blood at any one time.

From the above discussion it will be seen that although all the investigators are impressed with the importance of the thoracic duct as an avenue for the supply of lymphocytes to the circulating blood, yet no one has made a consistent attempt to analyze the changes that take place in the animal economy following ligation of the thoracic duct in order that the normal number of lymphocytes be again restored to the blood stream; nor has any one determined the approximate time following ligation of the duct at which a collateral circulation of the thoracic duct has mediated in the establishment of the normal preligation supply of lymphocytes to the blood stream.

Whether lymphocytes may enter the blood stream directly at their site of formation, as in the lymph glands and spleen, is another factor that enters into the problem. According to von Schumacher, who has studied the lymph glands in this respect, the blood in the vein leaving a lymph gland has more leucocytes than that in a neighboring artery. Unfortunately he did not study the blood in the artery going to the lymph gland in question; also, no figures were given to indicate the extent of the difference in the number of leucocytes in the afferent and efferent blood vessels.

Besides the definite decrease in the number of lymphocytes following ligation there were striking changes in other cell groups. The polymorphonuclear neutrophils invariably increased greatly in number immediately after the operation, but then gradually returned to a normal level. Contrariwise, the eosinophils decreased in number following operation but soon returned to their preligation level. The number of mononuclears and transitionals did not seem to have been influenced much by the operation.

The hematology of the laboratory animals does not seem to have been studied very extensively. Burnett, Busch and Van Bergen, Goodall, and Klieneberger and Carl all give figures for the differential counts of cat blood. Oddly enough the normal differential counts from Cat 1 corresponded to those of Goodall, while the counts from Cat 2 resembled those of Klieneberger and Carl. The latter were unable to find any mast cells in the blood.

SUMMARY.

This report has attempted to analyze the changes in the absolute number of small lymphocytes in the blood stream of the cat following the intrathoracic ligation of the thoracic duct. Such a ligation produced an immediate decrease in the number of small lymphocytes to the extent of 56 per cent, but it was found that the preoperative level was again reached at about the end of 3 weeks. One is led to believe that the gradual return of the number of small lymphocytes to the preligation level took place *pari passu* with the establishment of the collateral circulation of the thoracic duct, although there is no absolute proof of this. Yet it is definite that the thoracic duct is an important avenue for the entrance of small lymphocytes into the blood

stream, and that it is the pathway through which at least half of the small lymphocytes reach the circulating blood in the cat.

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THE TETANUS BACILLUS AS AN INTESTINAL SAPRO-PHYTE IN MAN.*

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It is well recognized that *Bacillus tetani* is widely distributed in nature, but the rôle of man as a carrier of this organism has received little attention. In the literature it is repeatedly stated that man may carry the tetanus bacillus in his digestive tract, the statement being apparently based on the work of Pizzini and also on the fact that this offers the best explanation for some idiopathic cases of tetanus and for the cases following typhoid fever, dysentery, and hemorrhoid operations.

Pizzini (1) injected suspensions of feces from 100 individuals into guinea pigs and in five instances obtained symptoms of tetanus. Ten of the individuals were hostlers, and the feces of three of them produced tetanus symptoms, while of the remaining 90, who were peasants, the feces of only two produced these symptoms. The natural conclusion drawn from this work was that man seldom carried the tetanus bacillus and that those who are in intimate contact with animals that are known to be carriers of this organism were more apt to be infected. During his extensive work on tetanus Tulloch (2) became interested in the digestive tract as a source of infection in abdominal tetanus. He studied the feces of twenty-one men returned from overseas service and in seven, or 33 per cent, isolated tetanus bacilli. From the feces of thirty-one civilians in England he obtained this organism in five instances, or 16 per cent. Tulloch's results are surprising not because they differ from Pizzini's, for his methods were undoubtedly better, but because they show such a large percentage of infections of the human intestinal tract. It is difficult to explain why in the numerous studies that have been made on the anaerobic flora of the human digestive tract we find no mention of the isolation of tetanus bacilli, but this may be due to interest in other organisms rather than to the fact that these bacilli were absent.

* Reported in part during the Opening Exercises of the Peking Union Medical College, September 22, 1921.

Before Tulloch's paper came to our attention we became interested in the presence of tetanus bacilli in the digestive tract through a case on the service of Dr. A. S. Taylor in which the best explanation of the source of infection seemed to be a fecal contamination of a bed-sore. In the present paper we wish to report the results of the examination of the feces of 78 individuals and to bring out some evidence which makes us feel that tetanus bacilli are the normal inhabitants of the intestinal tracts of a certain number of individuals.

We should like to emphasise the fact that the examinations were, with one exception, made on the feces of male Chinese, and that they, as did Tulloch's overseas men, live in more intimate contact with the soil than does the average man in Europe or America, so that our findings may not be duplicated in the Occident. While the individuals examined were hospital patients, we tried to secure specimens from recently admitted cases on both the medical and surgical wards and the results would probably be the same if normal individuals were examined.

Methods.

The stools were collected in clean but not sterilized bedpans and a portion was transferred to sterile Petri dishes which were sent to the laboratory. Approximately a 10 per cent suspension was made in sterile salt solution, the cotton plug was pushed down, and a rubber stopper inserted into the mouth of the tube. The tube was then immersed in a water bath at 80°C. and kept there for 20 minutes to destroy the non-spore-bearing organisms, after which 1 cc. of the suspension was transferred to sugar-free bouillon containing a piece of sterile rabbit kidney or spleen in a fermentation tube as recommended by Smith (3) for the cultivation of anaerobes. After 4 days incubation films were made from the sediment in the tube and searched for forms characteristic of tetanus bacilli. If the terminal round spore-bearing forms were not found the stool was called negative, while if they were found the stool was not called positive until the bacilli had been isolated and it was shown that a spasm-producing toxin was formed that was neutralized by tetanus antitoxin.

The organisms were isolated by heating the impure cultures to 80°C. for 20 minutes transferring to sterile bouillon plus tissue in a fermenta-

tion tube, and plating when the tetanus-like forms predominated. This procedure was repeated until what appeared to be a pure culture was obtained, when it was plated once more and transplants were made from well isolated colonies. This apparently pure culture was grown in sugar-free bouillon plus tissue, in a fermentation tube, for 10 days, the bulb fluid was discarded, and the branch fluid was centrifugalized until it was clear. Field or white mice were then inoculated subcutaneously with this clear fluid, two receiving 0.001 cc., two 0.01 cc., two 0.01 cc. plus approximately one unit of tetanus antitoxin, and two 0.1 cc. plus the same amount of antitoxin. All of the cultures that we have called *Bacillus tetani* produced characteristic spasms and death in the mice injected with 0.001 cc. and in the mice injected with 0.1 cc. plus approximately one unit of tetanus antitoxin there were no spasms and the mice lived at least 5 days, when the experiment was discontinued.

Discussion of Methods.

This method involved a great deal of work as it was often very difficult to get the organisms in pure culture though they were plated repeatedly. The injection of mixed culture was tried and it was found that at times spasms and death did not occur when characteristic tetanus-like organisms were present although when these organisms were isolated they proved to be tetanus bacilli. The results of the injections of impure cultures made on 2 different days are given in Table I.

It will be seen that some of the mice died so early that a diagnosis could not be made, though spasms might have been obtained in these animals had smaller amounts been injected. Other mice showed spasms and a definite diagnosis could be made, but the animals inoculated with seven out of the sixteen cultures lived 5 days or more and showed no symptoms of tetanus, though it was afterwards proved that the culture with which they were inoculated contained tetanus bacilli. It is evident, therefore, that there are organisms which interfere with the production of, or destroy tetanus toxin and that the injection of mixed cultures is not a reliable method to use in the detection of tetanus bacilli.

TABLE I.

Effect of the Injection, into White Mice, of the Centrifugalized Branch Fluid from Fermentation Tubes Containing Sugar-Free Bouillon Plus Tissue Inoculated with Heated Suspensions of Feces and Incubated 10 Days.

The sediment from all of these tubes showed organisms having the morphology of tetanus bacilli and from all of them *B. tetani* was later isolated in pure culture.

Patient No.	Mouse.	Effect of the injection of 0.5 cc. of culture.	Result.
340	A B	Dead next morning. Cause? " " " " ?	?
411	A B	" in 20 hrs. Generalized tetanus. Died immediately after inoculation.	+
346	A B	Dead in 20 hrs. Generalized tetanus. " " 20 " " "	+
399	A B	" " 48 " " " " " 48 " " "	+
226	A B	Alive at the end of 2 wks. No spasms. " " " " " 2 " " "	-
231	A B	Dead in 48 hrs. Generalized tetanus. " " 48 " " "	+
317	A B	Alive at the end of 2 wks. No spasms. " " " " " 2 " " "	-
266	A B	Dead in 20 hrs. Generalized tetanus. " " 20 " " "	+
227	A B	" next morning. Cause? " in 24 hrs. Generalized tetanus.	+
172	A B	Alive at the end of 2 wks. No spasms. " " " " " 2 " " "	-
1426	A	Dead in 24 hrs. Generalized tetanus.	+
1496	A	Alive at the end of 2 wks. No spasms.	-
1456	A	" " " " " 2 " " "	-
1495	A	Dead in 48 hrs. Generalized tetanus.	+
1484	A	" " 5 days. No spasms. Death was probably due to an organism other than tetanus.	-
1494	A	Alive at the end of 2 wks. No spasms.	-

Bengtson and McCoy (4) have shown that the direct inoculation of animals with smallpox vaccine containing tetanus bacilli is not nearly as delicate a test for this organism as the injection of 10 day fermentation tube cultures, and our results show that this cultural test cannot be relied upon, at least when testing for tetanus bacilli when other intestinal organisms are present.

As all the earlier work on the presence of tetanus bacilli in the intestinal tracts of various animals and in soil is based either on results obtained from direct inoculation or the injection of impure cultures, it must be assumed that if the work were repeated and tetanus-like organisms isolated, the percentage of positive findings from street dirt, cattle, horse feces, etc., would probably be greater than those already reported.

We have made only one culture from each individual and only 0.1 gm. of stool was cultured, and we have relied upon the microscopic examination of the sediments from the fermentation tube cultures for the first separation of positive from negative stools. We therefore feel that our percentage of positive cases may be too low, but in a paper to be published shortly we shall show that from the blood evidence can be obtained of an intestinal infection, and that the blood findings and stool examinations, as we have made them, agree very closely.

With regard to the microscopic examinations it is worthy of note that in over thirty stools in which we found tetanus-like organisms on the first examination in only one instance did we fail to isolate organisms that produced toxin. We obtain the sediment from the fermentation tube for microscopic examination by introducing a capillary pipette down to the bend of the tube, thus obtaining more of the anaerobes than it is possible to get with a platinum loop. Practically all of the film examinations were made after the tubes had been inoculated 4 days, but lately it has been found that tetanus bacilli are more numerous in tubes that have been incubated 5 or 6 days. In examining films we rely mainly on the morphology of the organisms and pay little attention to the Gram staining, as on the 4th or 5th day most of the tetanus bacilli are negative to Gram's stain.

The results of the microscopic examinations have been so consistent that it is strongly urged that films should be made from anaerobic

cultures of vaccine virus on the 5th or 6th day of incubation. If organisms having the morphology of tetanus bacilli are found in these films a negative result following the injection of the mixed culture into animals should not be taken to indicate that tetanus bacilli are absent but the tetanus-like organisms should be isolated and tested for toxin production before the virus is used on man.

Results of the Examination of Stools from 78 Individuals.

Using the above methods we examined during April and May, 1921, forty-three stools of patients in the hospital and from eleven, or 25.6 per cent, obtained tetanus bacilli. In August, September, and October of the same year we examined thirty-five more stools and from sixteen, or 45.7 per cent, isolated tetanus bacilli. In all we have therefore examined 78 stools and from 34.7 per cent have obtained organisms which in their morphology and toxin-producing properties were tetanus bacilli.

An examination of the histories of the twenty-seven positive cases reveals little that bears on the problem. With the exception of one American they were all male Chinese of the poorer class. The youngest was 5 and the oldest 44 years, and their average age was 20.3 years. Twelve of them had been in the hospital less than 10 days and eight more than 30 days. They had a variety of diseases, and from our point of view they may be taken to represent the average Chinese of the lower class.

While our results might indicate that carriers of tetanus bacilli are more numerous at certain seasons of the year we feel inclined to believe that the higher percentage of positive cases in the last group is due to the fact that we were more expert in the examination. We should therefore prefer to group our cases together and to say that in Peking among the lower classes at least one out of every three individuals is discharging tetanus bacilli in his feces.

Our results agree with Tulloch's findings in overseas men, and we predict that similar results will be obtained in other regions where great numbers of people live in close contact with the soil. In Peking this contact is very intimate, for during the winter and spring it is very dry and windy so that dust from the streets as well as from the fields outside the city (fertilized with human feces) is constantly being taken into the gastrointestinal tract.

Tetanus Bacilli as Intestinal Saprophytes.

We naturally became interested in the much disputed question as to whether the tetanus bacillus is primarily an inhabitant of the digestive tract as claimed by Sormani, or whether Vincent's contention is correct that its main habitat is outside the body and that spores found in the feces are those taken in with the food that have passed through the tract without germinating.

Sormani (5) based his contention on the facts that he found tetanus bacilli in the feces of a variety of animals, and in a dog infected by feeding, the feces showed tetanus spores 16 days later though it was fed on sterile food and was prevented by a muzzle from taking other food or licking contaminated surfaces. He reasoned that conditions for growth were more favorable in the body than outside and concluded that the former was its natural habitat. Vincent (6) admitted that tetanus spores might be found in the digestive tract but he failed to find them. He also showed that there was a destruction of the spores when they were introduced into the stomachs of rabbits or guinea pigs whose pylori had previously been ligated, and that the intestinal secretions prevented the development of tetanus bacilli in the test-tube. He also failed to find evidence of multiplication of tetanus bacilli in the intestinal tract of rabbits 12 hours after feeding tetanus spores. Since he was unable to show that tetanus bacilli multiplied in the digestive tract he concluded that their natural habitat must be outside the body. Bombicci (7) and von Esmarch (8), however, found that tetanus bacilli disappeared from putrefying mixtures, which is against Vincent's view that they are saprophytic organisms. Noble (9) examined the feces of a horse, a natural carrier, over a period of 7 months during the first 4 months of which time tetanus spores were found on every examination, while two negative horses in the same stable remained negative during this period though they were receiving the same food.

We had rather exceptional facilities for studying the question as to whether tetanus bacilli multiplied in the digestive tract as the patients in the hospital were receiving practically sterile food. The reducing valves for the steam-cookers had not been installed so that the steamed bread and rice that were the principal articles of their diet were cooked under a pressure of from 30 to 50 pounds of steam. The vegetables were carefully washed, peeled, and then boiled for at least 20 minutes. While a few tetanus spores might survive the latter process this is hardly likely, and we felt that if we could show that patients who had been in the hospital for some length of time eliminated spores, this would indicate that the organisms were multiplying in their intestinal tracts.

While the hospital food is practically sterile the patients' friends undoubtedly break the rules of the institution and bring in food from the streets which might contain tetanus spores. This is a factor that could not be controlled but the chances of infection are much less than in the recently admitted patients.

Eleven stools were received from patients that had been in the hospital for some length of time. The results of the examination are given in Table II where it will be seen that from seven of these eleven cases, or 63.6 per cent, that had been in the hospital 23 days or longer tetanus

TABLE II.

Presence of Tetanus Spores in the Stools of Patients Who Had Been Receiving Practically Sterile Food for a Considerable Time.

August 9, 1921.

Patient No.	Length of time in hospital.	Tetanus spores in feces.
	<i>days</i>	
231	36	Positive.
299	27	Negative.
317	23	Positive.
266	30	"
227	39	"
172	45	"
152	47	"
144	28	Negative.
226	41	Positive.
238	40	Negative.
138	59	"

Patient 172 was an American.

bacilli were isolated from the feces. In addition, the bacillus was found in a case that has been in the hospital 105 and in another that has been in the hospital 86 days. Our series of eleven cases is so small that we do not care to emphasize the fact that the percentage of carriers (63.6 per cent), is nearly twice that found in our complete series (34.7 per cent), but these results seem to indicate that the tetanus bacilli are multiplying in the digestive tracts of these patients.

We next tried to estimate the number of tetanus spores in the stools of three carriers by making known dilutions of the stools and determining the highest dilution in which tetanus bacilli could be found.

The stools were received in sterile bedpans, weighed, mixed, and from accurately weighed portions 10 per cent suspensions were made in sterile salt solution. These suspensions were shaken vigorously to break up clumps and a portion was transferred to other sterile test-tubes that were corked and then heated to kill the non-spore-bearing organisms. A series of known dilutions was then made and from each 1 cc. was transferred to each of two fermentation tubes containing sugar-free bouillon plus tissue. After 4 days incubation films were made and examined for tetanus-like organisms. From the tube containing the highest dilution of feces that showed tetanus-like organisms anaerobic plates were made and in each case pure cultures were obtained that produced toxin which caused the characteristic

TABLE III.

Estimation of the Approximate Number of Tetanus Spores Eliminated by Three Individuals.

Patient No.	Age.	Diagnosis.	Length of time in hospital.	Weight of stool.	No. of tetanus spores.	
					Per gm. of feces.	Eliminated.
	yrs.		days	gm.		
158	5	Kala-azar.	105	74	10,000	740,000
374	9	Tubercular osteomyelitis.	35	258	10,000	2,580,000
932	13	Tuberculosis.	27	200	1,000,000+	200,000,000

symptoms of tetanus in mice and which was neutralized by tetanus antitoxin.

The results obtained from the examination of these three cases are given in Table III. It so happened that children were chosen for the test so that their stools were small, but it is inconceivable that this great number of spores was taken into the intestinal tract and passed through without germinating. The tetanus bacilli must have been established and growing in the intestinal tract.

DISCUSSION.

A discussion of the methods used in detecting tetanus bacilli in mixed cultures is to be found in the body of the paper and will not be repeated here. It is sufficient to say that the isolation of the bacillus and the demonstration that the culture forms a spasm-producing toxin

that is neutralized by tetanus antitoxin is the only reliable method. The injection of mixed cultures gives uncertain results, for while tetanus bacilli may be present, other organisms may interfere, with toxin production.

The results show that one-third of the male population in the vicinity of Peking carries tetanus bacilli in the digestive tract and that the bacilli probably are multiplying there. Man thus plays a large rôle in the distribution of the bacillus, for it is not uncommon to see human feces deposited on the streets, and human feces are used to fertilize the fields, so that dust from the streets and fields must contain great numbers of spores that come from man. One wonders why all of the population are not carriers of the bacillus, and it may be that at some time in their life they are but that other organisms crowd the tetanus bacillus out or make conditions unfavorable for its growth.

It is difficult to say just how these large numbers of tetanus carriers influence the case incidence of tetanus. Foreign doctors see very few cases of infection, probably not more than in European countries. In our own hospital with a large out-patient clinic and nearly 2,000 in-patients per annum there were six cases of tetanus in 1919 and three cases in 1920, and physicians who have practised in China for years have told us that they rarely see cases of tetanus. We have been unable to obtain a record of tetanus following typhoid fever or dysentery, though these diseases are extremely common. Rose (10), however, reports several cases of tetanus following typhoid in Germany, and there are numerous reports in the literature of cases following hemorrhoid operations, so that it is evident that the bacilli in the digestive tract are at times able to enter the body and produce their toxin. We have seen one case of tetanus following a fall that ruptured the intestine but did not produce an external injury. It is evident, however, that foreign physicians in China see in adults very little tetanus due directly or indirectly to an intestinal infection. This may be owing to the fact that the Chinese recognize the disease as a fatal one and that patients are afraid to come to foreign hospitals as they want to die at home. On the other hand, it may be that carrying the tetanus bacillus in the digestive tract for some time produces a relative immunity to this organism. In a paper to be published shortly we shall produce evidence which supports this latter view.

While we have no accurate statistics we think that tetanus neonatorum is extremely common. Lennox (11) in questioning hospital patients as to their past history found that of 8,468 children born, 61 per cent died of convulsions or madness. It is quite probable that many of these deaths were due to tetanus, the infection coming from the filthy dressings used on the cord, and while the figures are only approximate they give some indication of the great loss of life which is in part, at least, associated with the spreading of tetanus bacilli by man.

CONCLUSIONS.

1. The only reliable method that can be used for the detection of tetanus bacilli is the culturing of the suspected material, the isolation of tetanus-like organisms and the demonstration that the pure cultures form a spasm-producing toxin that is neutralized by tetanus antitoxin.

2. Using this method we have demonstrated tetanus bacilli in 34.7 per cent of stools from 78 individuals in Peking.

3. The tetanus bacillus is growing in the digestive tract, for it is present in individuals who have been on a practically sterile diet for a month or more, and one individual may eliminate several million spores of tetanus bacilli in a single stool.

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AN EPIDEMIOLOGICAL STUDY OF RHINITIS (CORYZA) IN CALVES WITH SPECIAL REFERENCE TO PNEUMONIA.

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During the months of November and December, 1921, an outbreak of pneumonia occurred among calves in a dairy herd. This herd has been under observation since 1917 and from previous studies the organisms associated with pneumonia have been established. Theobald Smith¹ in 1917 noted cases of pneumonia among the calves. During 1919 he studied twelve cases of calf pneumonia. In nine instances *Bacillus actinoides* was found. *Bacillus boviseplicus* was also associated with three or four of these. During the latter part of October and throughout November, 1920, there occurred a severe outbreak of pneumonia² in adult cows which had been shipped from Michigan. The disease spread to other newly purchased cows and to local and native stock. The outbreak subsided in the dairy herd but cases were observed among the native calves. A few calves born of the Michigan cows had been taken to the calf barns. It was assumed that these calves may have carried the virus.

It has been possible by microscopic and bacteriological examination of the nasal exudate of calves to throw some light on the means of dissemination of the virus and the method by which it may be maintained over considerable periods.

In previous publications Jones^{3,4} was able to show that the organisms associated with the outbreak in the cows and infections in calves

¹ Smith, Theobald, *J. Exp. Med.*, 1921, xxxiii, 441.

² Jones, F. S., and Little, R. B., *J. Exp. Med.*, 1921, xxxiv, 541.

³ Jones, F. S., *J. Exp. Med.*, 1921, xxxiv, 561.

⁴ Jones, F. S., *J. Am. Vet. Med. Assn.*, 1921, lx, 271.

occurring throughout the late fall of 1920 and the early months of 1921 differed from those obtained by Theobald Smith during 1917, 1919, and 1920. For convenience they may be referred to as *Bacillus bovisepiticus* Groups I and II. Both types are morphologically similar. The groups may be characterized as follows:

Group.	Acid production in.					Hemolysis.	Bile solubility.	Indole.
	Dextrose.	Lactose.	Saccharose.	Maltose.	Mannitol.			
I								
Associated with outbreak in 1920 and sporadic cases during 1920 and 1921	+	+	+	+	+	+	-	-
II								
Associated with <i>actinoides</i> pneumonia in 1917, 1919, and 1920	+	-	+	-	-	-	+	+

Immunological grouping by means of agglutination affinities followed the cultural grouping. The serum of a rabbit injected with a single Group I strain agglutinated all the Group I cultures but failed to affect those of other groups. A specific agglutinin was also produced for Group II. In no instance was cross-agglutination observed.

It has been stated that during November and December, 1921, there occurred in the calves a small epidemic of pneumonia. Calves which presented no symptoms of pneumonia were frequently found to be suffering from a coryza accompanied by a mucoid or mucopurulent discharge. A number of those showing characteristic symptoms of pneumonia also exhibited a purulent nasal discharge. The discharge from several was examined and organisms possessing all the characters of Type I were obtained. The findings warranted a more complete study. Examinations of the nasal exudate of all the calves in the barn were undertaken.

Method of Examination.

A sterile swab was inserted into both nostrils for a distance of 6 to 8 cm. Films were then prepared on sterile cover-slips. The swab was agitated briskly in 2 cc. of sterile NaCl solution. From this

suspension blood agar plate cultures were made. At first mice were injected beneath the skin with small amounts of the suspension and cultures made from lesions developing about the site of injection. The results were on the whole of less value than those obtained by the plate method, especially since Group I possesses little pathogenicity for any of the laboratory animals. The hemolytic properties of Type I in blood agar plate cultures render its recognition easy. In addition, it is possible to formulate a fair idea of the proportion of *boviseppticus* to other organisms. That the methods employed are not such that it would be possible to detect *Bacillus boviseppticus* in small numbers is admitted, but it was necessary to utilize the material as quickly as possible especially as young calves, probably bringing new infections, were constantly introduced into the barns.

Colonies surrounded by the narrow zone of hemolysis were fished from the plate cultures. If on the microscopic examination the subcultures resembled *Bacillus boviseppticus*, transfers were made to plain agar. After suitable incubation the growth was suspended in 0.85 per cent NaCl and tested against Group I serum. When a culture agglutinated with the serum the fermentation characters were defined.

Material obtained on the swabs must be utilized as quickly as possible. The moist swabs standing at room temperature soon become overgrown with bacteria. Even when swabs are stored in the refrigerator the number of specific organisms diminishes rapidly.

Result of Examinations.

A diagram of the arrangement of the calves in the barn will serve to illustrate the proportion of calves suffering with pneumonia and those which were carrying *Bacillus boviseppticus* in the nasal passages (Table I). The pens are separated from each other by an iron fence with spaces 4 or 5 inches between the uprights.

It will be noted that of the thirty-two calves originally in the barn, two died of pneumonia. In both instances typical organisms of Type I in pure cultures were isolated from the consolidated portions of the lung. In these cases bacteriological examinations of the nasal passages were not made. Of the remaining thirty calves, eight developed clinical manifestations of pneumonia. Of these, four showed a characteristic nasal discharge from which Group I organisms were

TABLE I.
Arrangement of Calves in Pens during the Outbreak.

1. Calves 3 to 4 wks. old.	2. Calves 1 mo. to 5 wks. old.	3. Vacant.	4. Calves 2 to 3 mos. old.	5. Calves 3 to 4 mos. old.	6. Calves 4 to 5 mos. old.	7. Calves 6 mos. old.
1236 Rhinitis. 1237 1238 Rhinitis.	1231 Pneumonia. 1232 1233 Pneumonia; rhinitis. 1234 Rhinitis. 1235 " 675 Pneumonia (died).		1226 1227 Pneumonia. 1228 1229 Pneumonia; rhinitis. 1230 Rhinitis. 676 Pneumonia (died).	1221 1222 Rhinitis. 1223 Pneumonia; rhinitis. 1224 1225	1215 Pneumonia; rhinitis. 1216 1217 Rhinitis. 1218 Pneumonia. 1219 Rhinitis. 1220 Pneumonia.	1209 1210 1211 1212 1213 1214 673 Rhinitis (in- troduced Nov. 18, 1921).

cultivated. Eight other calves failed to show symptoms of pneumonia but developed a mucopurulent nasal discharge. From these exudates Type I organisms were cultivated. It will be noted that the oldest calves in Pen 7 all remained free from pneumonia and none developed rhinitis. Calf 673 will be referred to later when certain of the experimental observations are discussed.

The general symptoms of the calves with pneumonia have been noted in a previous communication. There is little disturbance in the general health of calves suffering only from the nasal infection. The temperature may be slightly above the normal. The appetite remains good and there is no apparent digestive disturbance. The calf may occasionally cough or sneeze, forcing out a variable quantity of exudate. The exudate is composed of mucus and masses or strings of polymorphonuclear leucocytes. *Bacillus bovissepticus* is found in varying numbers, often making up from 80 to 90 per cent of the organisms in the plates. At times there was no visible exudate about the nostrils. The introduction of the swab, however, may induce sneezing and lead to the expulsion of exudate. In many instances the nostrils are kept clean by licking so that on casual examination exudate was not observed although the calf suffered with a more or less severe rhinitis.

It is customary on this farm to group calves of the same age in one pen as far as practicable. After they have reached a certain age they are removed to another barn. So a steady stream of calves progresses through each barn. The barn in which the outbreak occurred was quarantined during the epidemic. After the disease had subsided young calves, the majority of which had been injected twice with killed cultures of Group I, were introduced according to routine. During the epidemic board partitions had been erected between certain pens but there remained ample opportunity for contact infections. The next twenty-four calves introduced into this barn were examined at frequent intervals. Among the first lot of vaccinated calves introduced one was suffering from rhinitis. Plate cultures failed to show the hemolytic colonies characteristic of Group I, although a large proportion of the colonies were similar. Suspicion was at once aroused that another group had been introduced. Such proved to be the case. A typical Group II organism was identified.

From this time onward practically all calves placed in this barn developed a rhinitis. In twenty-one instances cultures which fall into Group II have been obtained.

It has been found that the solubility in ox bile of this class of organisms has been of assistance in making identifications. As soon as these organisms were recognized, killed cultures of Group II were included in the vaccine. The nasal infections with Group II continued and were apparently not influenced by the vaccine.

Group I organisms reappeared and were recognized in certain of the newly introduced unvaccinated calves 36 days after the quarantine was lifted. A fatal case of pneumonia developed and two cases of rhinitis occurred. The source of this infection is obscure.

EXPERIMENTAL.

Although the bacteriological and microscopic findings indicated the presence of *Bacillus bovisepiticus* within the nasal passages associated with a characteristic purulent exudate, the possibility of other organisms as the primary etiological agent could not be excluded. More complete studies than those made on the spontaneous cases were desirable. With this in view a number of experiments were undertaken.

Experiment 1.—Calf 698. Born Dec. 6, 1921. Dec. 21. The mucosa of both nasal passages was brushed with sterile swabs, films and salt solution suspensions for plate cultures were made from each swab. The films when stained with methylene blue failed to show leucocytes or organisms resembling *B. bovisepiticus*. Methods similar to those employed in the herd observations were used in the experimental studies. It was felt that by suspending the material from the swabs in the same amount of salt solution at each examination and using the same amount of the suspension for the preparation of the plates an adequate idea of the increase or decrease of the organisms could be obtained. The cultures used for inoculation were all isolated within a month. They had been passed through three successive passages on horse blood agar.

Dec. 22, 5.15 p.m. The septum of the right nasal passage was brushed with a sterile swab which had been dipped in an 8 hour bouillon culture and then brushed over the surface of a blood agar culture of *B. bovisepiticus* (Type I) obtained from the nasal passages of a calf.

Dec. 23. The calf appeared well. There was no visible discharge from the nostrils. Insertion of a sterile swab into the right nostril induced sneezing. A considerable mass of yellowish white, viscid material was ejected. The material

was composed largely of densely packed masses of leucocytes. Characteristic bipolar encapsulated rods were observed in the stained films. Blood agar plate cultures from the suspension revealed a moderate number of Type I colonies in practically pure culture. Material from the left nostril failed to show either leucocytes or *B. bovisepiticus*. The highest temperature recorded on this day was 39.1°C.

Dec. 24. There was considerable purulent discharge from the right nostril. Stained films revealed masses of leucocytes and characteristic *bovisepiticus* forms in large numbers. The plate cultures revealed numerous characteristic colonies. Films from the left nostril revealed a few leucocytes. The plate cultures did not show colonies of *B. bovisepiticus*. The temperature was normal.

Dec. 25. Considerable purulent discharge from both nostrils was noted. The films from the right nostril revealed leucocytes in enormous numbers. The plate cultures showed more colonies than on the preceding day. Preparations from the left nostril contained leucocytes in considerable numbers. In certain instances polymorphonuclear leucocytes which had taken up characteristic rod-like organisms were encountered. Colonies of *B. bovisepiticus* did not develop in the plate cultures. The temperature varied between 38.5° and 38.8°C.

Dec. 26. Purulent exudate from both nostrils. *B. bovisepiticus* in large numbers in plate cultures from the right nostril.

Dec. 26 and 27. A maximum temperature of 40°C. was recorded.

Dec. 28. The condition was unchanged. About the same number of colonies of *B. bovisepiticus* in the plates from the right nostril. After this date the temperature gradually declined until the normal was reached.

Jan. 3, 1922. Right nostril showed purulent exudate. Film showed leucocytes in great numbers and a moderate number of the organisms. Plate cultures showed about the same number of colonies observed Dec. 28. There was no visible exudate from the left nostril. Films revealed very few leucocytes. The plates were negative.

Between Jan. 3 and 10, the amount of exudate from the right nostril gradually diminished. On Jan. 10, material obtained from this nostril contained only an occasional leucocyte. *B. bovisepiticus* was not observed in the films nor obtained from plate cultures.

Jan. 11. The calf was reinoculated in the left nostril with the same culture. A purulent discharge was observed on Jan. 12. *B. bovisepiticus* was observed in the films and plate cultures but in small numbers. The nostrils were examined daily for the next few days but *B. bovisepiticus* could not be obtained from the plate cultures.

During this experiment the general condition of the calf was unaffected. There was, however, a slight temperature disturbance during the height of the disease.

Experiment 2.—The preceding experiment was repeated. Jan. 11, 1922. Calf 697 (aged 43 days) was inoculated in the left nasal passage with a sterile swab dipped in culture. Within 24 hours there was considerable purulent discharge from both nostrils. The organisms were found in the exudate from both nostrils

the 2nd day after inoculation. The greatest numbers were recorded between the 2nd and 5th days. The last record of their presence in plate cultures was on the 12th day. Of interest is the fact that *B. bovisepeticus* appeared in the plates in relatively pure cultures. It is to be expected that a few air forms, streptothrix and molds, will be cultivated from the nasal passages of herbivora since they make up a considerable proportion of the organisms found in hay and straw. After the 12th day all examinations were negative. On the whole, the conditions seemed more severe in the case of this calf. The exudate was more copious. The general condition remained unchanged although slight temperature disturbances were recorded.

An opportunity was afforded for observations on spontaneous transmission in the instance of Calf 673 (Table I). The calf was known to be free from Type I infection when placed in a pen adjoining calves showing characteristic rhinitis. Within 3 days there was a slight temperature disturbance. Examination after 5 days showed the characteristic purulent discharge. Plate cultures from the nasal exudate revealed that 70 per cent of the organisms were Type I *bovisepeticus*. This calf never manifested symptoms of pneumonia. The organisms subsequently disappeared from the nasal passages.

These experiments clearly establish the findings within the herd that the rhinitis met with during the small outbreak of pneumonia may be attributed to infections with Group I *bovisepeticus*.

We have called attention to our failure to find Type I *bovisepeticus* in the nasal passages of freshly introduced calves after the outbreak had subsided. The rhinitis continued throughout the months of December and January but in nearly every instance organisms recognized as Group II were obtained. In a number of instances calves recently introduced into these pens would fail to show either leucocytes or *Bacillus bovisepeticus* II in the nasal secretion. A week later many of them would have developed a purulent nasal discharge and *Bacillus bovisepeticus* II would appear in the plates. Injection with a vaccine containing killed cultures of this organism failed to protect against such infections. To ascertain whether the organisms of Group II were capable of causing these manifestations, two calves were inoculated as in the preceding experiments. Pure cultures recently obtained from the nasal discharges were employed.

Experiment 3.—Jan. 26, 1922. The mucosa of the right nostril of Calf 715B (21 days old) was brushed with a swab immersed in an 18 hour bouillon culture and

then smeared over the surface of an 18 hour blood agar slant of a typical Group II strain.

Jan. 27. There was no visible exudate but films from the swab showed leucocytes in masses and a few *bovissepticus*-like forms. The plate cultures failed to show suggestive colonies. The films from the left nostril revealed only epithelial cells and a few cocci.

Jan. 28. There was no visible exudate. The swab from the right nostril showed purulent exudate. Leucocytes in masses and a moderate number of characteristic rods were present in the films. The plates revealed a considerable number of colonies, at least 90 per cent of which resembled *bovissepticus*. Material from the left nostril did not contain leucocytes or *B. bovissepticus*.

Jan. 30. The right nostril contained a small quantity of thick, yellow, purulent exudate containing leucocytes in masses and large numbers of characteristic rods. The plate cultures were largely made up of *B. bovissepticus* II. The secretion from the left nostril failed to contain leucocytes or characteristic organisms.

Jan. 31. There was purulent exudate on the swab from the right nostril. The organisms were more numerous in the plate cultures.

Feb. 2. There were leucocytes in the material obtained on the swab from the right nasal passage. The plate cultures showed only about half as many colonies as on Jan. 31.

Feb. 6. Relatively few leucocytes in the secretion from the right nasal passage. *B. bovissepticus* still made up 80 per cent of the colonies. The swab from the left passage failed to show leucocytes or characteristic colonies.

Feb. 10. *B. bovissepticus* still persisted in the right nostril. They were still present on Feb. 15.

Experiment 4.—Calf 757, 16 days old when inoculated in the left nasal passage with Type II culture obtained from the nasal exudate of a calf. In general the course of the infection paralleled that of Calf 715B. The exudate was purulent during the first 4 days. The organisms were most numerous 3 days after inoculation. After 5 days the exudate contained only a few leucocytes but still contained the organism. From the right nasal passage it was not possible to obtain *B. bovissepticus* in the plates.

Neither of these calves showed a systemic reaction. The temperature remained well within the normal limits.

From the experiments it appeared that Group I organisms when introduced into the nasal passages increase in number for 4 or 5 days. After this maximum is reached there is a sharp decline and in most instances the organism can no longer be cultivated after 10 to 18 days. With Group II the organisms persisted after the disease had subsided.

If it were possible to show that organisms of both groups were not entirely eliminated from the nasal passages in certain individuals

after considerable periods, it would be possible to explain the sudden appearance and disappearance of pneumonias in the herd. With this in view all calves which had suffered from rhinitis were reexamined during the latter part of February and early part of March, 1922. In two cases (Calves 1234 and 1235, Table I) Type I organisms were still present in the nasal passages 113 and 121 days after their first discovery. In another instance a calf infected during January, 1922, was known to have carried the organisms for a period of 36 days.

About the same proportion of calves continued to carry Group II organisms. Of sixteen calves reexamined, four continued to harbor Group II organisms within the nasal passages after periods of 50, 66, 66, and 73 days. It seems reasonable to conclude that certain calves may become carriers of both groups for indefinite periods. It must be borne in mind that the methods employed are such that carriers of small numbers of organisms may be overlooked.

DISCUSSION.

The observations are of considerable interest in certain respects. It is possible to divide the calves in the outbreak associated with *boviseppticus* Group I into four lots. Those that were most susceptible to the infection died of diffuse pneumonia. A larger number, more resistant perhaps, developed severe clinical manifestations and apparently recovered. Still others failed to develop any symptoms of grave respiratory disturbance but suffered from a coryza. These animals were exposed to the same general environment as the others but for some reason were more resistant. Scattered throughout the pens were other calves that failed to present any manifestations of infection. In addition to these there remain the oldest calves in Pen 7. Group I organisms were not found in the nasal passages nor was pneumonia observed among them. These calves were born in May and June, 1921. They may have been exposed to cases in these months and developed a certain degree of immunity. It is known that sporadic cases occurred in this barn during the spring of 1921.

Of greater interest is the ability of both types of organisms to exist in specific instances on the nasal mucous membrane. From the epidemiological point of view this is of considerable significance. Group I is capable of producing a severe pneumonia or a mild infection

of the nasal passages. Two calves which suffered only from the rhinitis have continued to carry these organisms in the nasal passages for several months. It is possible to explain the sudden outbreaks in calves in the winter months to exposure and infection from the mild cases of rhinitis or to carriers. The same explanation may be applied to some of the outbreaks among cattle which have been shipped. Often cows and steers for shipment are purchased from several sources. After they have been assembled they are loaded and shipped. The inclusion of a carrier or a mild case of rhinitis would afford ample opportunity for infection. This is especially true when the conditions of shipment are considered. There is ample opportunity for considerable lowering of resistance, especially during the colder months of the year.

Newsom⁵ in discussing the various forms of hemorrhagic septicemia in sheep describes an acute type (septicemic) and the more chronic types, such as the pulmonary, enteric, and cerebral forms. For the sake of completeness he calls attention to a chronic catarrhal disease of the nasal passages of sheep, although he states that he has no definite knowledge that it is a form of hemorrhagic septicemia.

There are few data relative to the pathogenicity of organisms belonging to Group II. In the observations on this particular herd they have not been regarded as the prime factor in pneumonias of either cows or calves. Further observations to establish their relation to pneumonias of calves are necessary. They have been found by Theobald Smith in calves infected with *Bacillus actinoides*. It has been possible to show that Group II is associated with a mild rhinitis which is communicable from infected to healthy calves.

Some of the characters possessed by both types render their recognition easy. The narrow hemolytic zones about the deep colonies of Type I in horse blood agar plates are an important character. After the study of many strains it was found that cultures of the proper morphology which were hemolytic and agglutinated with the Group I serum possessed the fermentation characters of the cultural group. Bile solubility affords a ready method of distinguishing the members of Group II. From present indications it seems safe to consider

⁵ Newsom, I. E., 23rd Ann. Rep. U. S. Livestock San. Assn., 1919, 203.

that, those organisms which are bile-soluble and agglutinate with Group II serum will fall into a specific cultural group.

Early in the course of the study it was hoped that it would be possible to type the organisms obtained by means of swabs from outbreaks occurring some distance from the laboratory. It was found that material obtained on the swabs rarely showed viable *bovisepeticus* types after 18 to 24 hours in the refrigerator. Material standing at room temperature was soon overgrown with other organisms and became valueless for diagnostic purposes.

SUMMARY.

During the month of November there occurred an outbreak of pneumonia among the calves in a large dairy. Thirty-two calves in one barn were exposed to the disease. Ten clinical cases developed. Two died of diffuse pneumonia. From these *bovisepeticus* Group I organisms were obtained at autopsy. Four affected with pneumonia and eight other calves which failed to show symptoms of pneumonia developed a purulent rhinitis. From the nasal exudate of these cases Group I organisms were cultivated. The characteristic rhinitis was reproduced experimentally by brushing the nasal mucosa with a swab dipped in culture. Certain of the calves which suffered from the spontaneous rhinitis continued to carry the organisms in the nasal passages for periods as long as 121 days.

After the first outbreak had subsided practically all calves introduced into this barn developed a milder type of rhinitis associated with organisms of Group II *bovisepeticus*. 25 per cent of such calves continued to carry the organism on the nasal mucosa for periods of 50 to 73 days. It was possible to induce nasal infection in calves with pure cultures of this organism.

MIXED CULTURES OF PURE STRAINS OF FIBROBLASTS AND EPITHELIAL CELLS.

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PLATES 15 TO 18.

(Received for publication, May 5, 1922.)

For years the question of dedifferentiation or transformation of tissue cells into an indifferent embryonic cell type when cultivated *in vitro* has been under discussion. Champy¹ cultivated kidney tissue, and found that its structure changed; indifferent tubules were formed which later lost their renal and finally their epithelial characteristics. From this and other experiments, he concluded that embryonic tissue cells cultivated *in vitro* dedifferentiate sooner or later, sometimes after a few hours. Uhlenhuth² showed that tapetum cells from the retina changed their typical epithelial character with modifications in the consistency of the culture medium. We found also that, when epithelial cells were allowed to grow embedded in the clot, their shape changed from polygonal to fusiform. Therefore, under these conditions, cultures of epithelial cells looked like cultures of fibroblasts, but under high magnification, there was no difficulty in distinguishing epithelial cells from fibroblasts. Although the epithelial cells proliferating in the clot are spindle-shaped, they have a peculiar way of growing close to each other (pavement-like), which is not true of fibroblasts. In a previous article,³ it was shown that epithelial cells in pure cultures still remained typical after more than 3 months. The morphological characteristics of the cells did not change as long as the strain was kept under constant cultural conditions; that is, on the free surface of the plasma clot under a film of embryonic tissue juice.

¹ Champy, C., *Bibliog. Anat.*, 1913, xxxiii, 184.

² Uhlenhuth, E., *J. Exp. Med.*, 1915, xxii, 76.

³ Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367.

In order to settle the question of dedifferentiation, it was thought of interest to determine whether epithelial cells and fibroblasts could be distinguished from one another after they had been allowed to grow side by side in the same culture for several generations.

I.

Technique.

Fragments of the strains of fibroblasts and epithelium were cultivated side by side under identical conditions (Fig. 1). After 48 hours, a distinct difference was observed in the character of the growth of the two fragments. The epithelium grew in a compact mass, with the individual cells in close contact. The fibroblasts migrated into the culture medium and formed a network. In the fixed preparations stained with methylene blue, it was not possible to determine definitely whether any amalgamation of the two cell types took place after the growth from both fragments had united. No more striking result was obtained by the Van Gieson method. Then, fragments of a 2 month old strain of epithelium and a 10 year old strain of fibroblasts were cultivated together for several generations. After a few passages, the fibroblasts overgrew the epithelial fragment, covering it completely. The combined culture showed a peripheral growth composed of fibroblasts, and the only apparent indication of the presence of epithelium was that the central portion of the culture appeared semitransparent and homogeneous, and not as dense and opaque as a typical culture of pure fibroblasts which had not been divided for the same number of generations. At this stage, the mixed cultures were divided and subcultures made. These in turn were allowed to grow for 48 hours, and were again divided through the central portion. This procedure was continued for seven generations and then the preparations were fixed, sectioned, and stained by Van Gieson's method. The sections showed typical epithelial and connective tissues, as found in the organism. The epithelium appeared greenish yellow in contrast with the connective tissue, which appeared pink and contained many fibrillæ which were stained a marked pink. No parts of the section showed an amalgamation of the two cell types. The epithelial cells were everywhere distinctly

differentiated. In many places a definite structural arrangement of the cell elements was observed (Fig. 2). The epithelial cells had grouped themselves to form tubules with distinct lumina. In several of the sections, the lumina could be seen filled with a homogeneous colloidal secretion (Fig. 3). The arrangement of the epithelial cells forming the tubules resembled the conformation found in sections of salivary glands. The individual cells which formed the tubules had their nuclei disposed close to the basal membrane.

In some parts of the section, epithelial cells could be seen penetrating the surrounding layer of fibroblasts and appearing on the free surface of the tissue fragment. In other parts, large masses of amorphous material (dead epithelial cells and secretions), surrounded by a layer of low epithelium, could be observed (Fig. 2).

II.

DISCUSSION.

These experiments show that epithelium cultivated for 2 months *in vitro* retained its morphological characteristics which differed decidedly from those of fibroblasts. But a still more striking fact was observed; namely, that the differential Van Gieson stain brings out the chemical difference between the two cell types when they are allowed to grow together. The epithelium was observed to have formative ability; *i.e.*, the epithelial cells arrange themselves in winding tubules. This has already been mentioned in an earlier report of experiments³ in which the epithelial cells, cultivated on the free surface of the clot, grew in a single layer and were described as organizing themselves in structures which closely resembled cross-sections of glands. The experiments herein described confirm this statement, since the tubular arrangement may be followed throughout serial sections.

Champy¹ states that no strains of cells can be cultivated for any length of time *in vitro* without a change occurring in their morphological characteristics. We have shown that fibroblasts⁴ and epithelial cells³ may be cultivated in pure cultures for long periods without dedifferentiation. Therefore, in this case it seems that Champy's

⁴ Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755.

statement does not confirm the experimental data. On the other hand, he also states that epithelial cells in the presence of connective tissue cells do not dedifferentiate. This fact is fully substantiated by the present work.

The foregoing experiments have made it possible to analyze more accurately the character of the growth of epithelium *in vitro*. When the fragment is cultivated upon the surface of a clot, the growth is characterized by the formation of a membrane, the individual cells are polygonal, and their growth resembles a pavement epithelium. This is the typical formation obtained by surface cultivation. When the fragment is cultivated in the clot, the growth seems to depend upon the existing condition within the medium and the disposition of the embedded fragment. When the cells are allowed to invade the medium uniformly, an extensive membrane is formed, very much like that obtained in surface growth (Fig. 4), but the individual cells are spindle-shaped (Fig. 5) and not polygonal. If the condition of the coagulum prevents the uniform outgrowth of new cells from the mother fragment, then the cell invasion is characterized by the formation of branching tubules of various forms, but essentially the arrangement of the growing cells is such as to form a more or less organized structure resembling hollow tubes (Fig. 6). The growth and migration of epithelial cells seem to be much more dependent upon the mechanical conditions than those of fibroblasts.

When growth occurs in membrane formation, it is rapid and extensive. When the tubular type results, the rate of growth is markedly slower and the actual increase in mass is small, although the length of the tubular outgrowth may be extensive.

Now that it has been proved that epithelium and fibroblasts cultivated *in vitro* remain two different types of cells, with individual characteristics, innumerable experimental possibilities are opened. It is obvious that the study of their respective interactions under different experimental conditions will lead to interesting findings.

III.

CONCLUSIONS.

1. Strains of epithelium and fibroblasts cultivated side by side in the same medium keep their individual characteristics. When sec-

tioned and stained by the Van Gieson method, the cultures show the epithelium stained greenish yellow and the fibroblasts and their fibrillæ pink.

2. There are no transition forms between the epithelial cells and fibroblasts.

3. The epithelial cells belonging to an older strain are still able to form primitive structures of winding tubules, with typical glandular epithelium.

4. Under the conditions of the experiments, no dedifferentiation takes place.

EXPLANATION OF PLATES.

PLATE 15.

FIG. 1. Experiment 1532-4. Culture of fibroblasts and epithelial cells cultivated together, after 48 hours incubation. Stained with methylene blue. *A*, fragment of a 10 year old strain of fibroblasts; *B*, fragment of a 2 month old strain of epithelium. \times about 20.

FIG. 2. Experiment 1387-1. Section through a mixed culture of a 10 year old strain of fibroblasts and 2 month old strain of epithelium. The preparation was fixed and stained by Van Gieson's method after having undergone seven passages *in vitro*. *A*, glandular arrangement of epithelial cells surrounded by fibroblasts; *B*, network of fibroblasts and bundle of fibrillæ; *C*, an area of degenerated epithelial cells surrounded by low epithelium. \times 120.

PLATE 16.

FIG. 3. Experiment 1387-1. Another section through the same culture as Fig. 2. *A*, glandular arrangement of epithelium; *B*, lumina; *C*, a lumen filled with secretion; *D*, waved connective tissue fibrillæ; *E*, characteristic position of the nucleus in the cell as it appears in secretory epithelium. Stained by Van Gieson's method. \times 400.

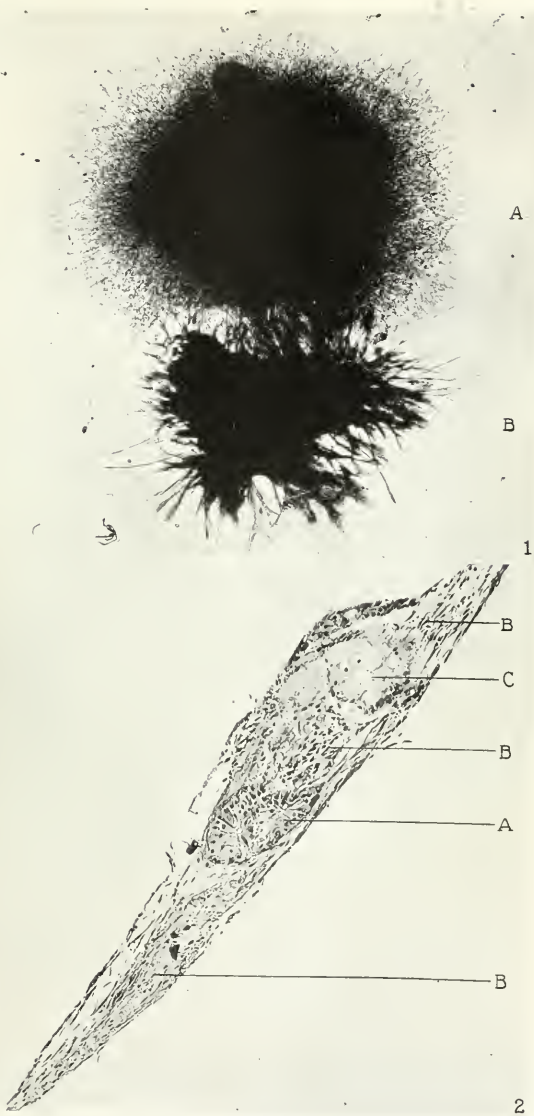
PLATE 17.

FIG. 4. Experiment 25970-2. Seventeenth passage of a pure culture of epithelium growing in a membrane. Fragment cultivated embedded in the clot. Fixed and stained with Azur II after 48 hours incubation. \times about 80.

PLATE 18.

FIG. 5. Experiment 25970-2. Same preparation as in Fig. 4. The individual cells appear spindle-shaped and flat. \times about 200.

FIG. 6. Experiment 1128-2. Thirteenth passage of a pure culture of epithelium growing embedded in the clot. The new growth appears as solid processes and tubules. Stained with Azur II. \times about 160.



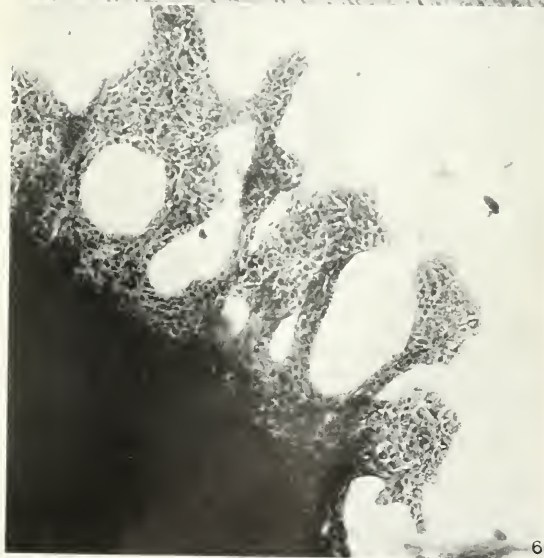
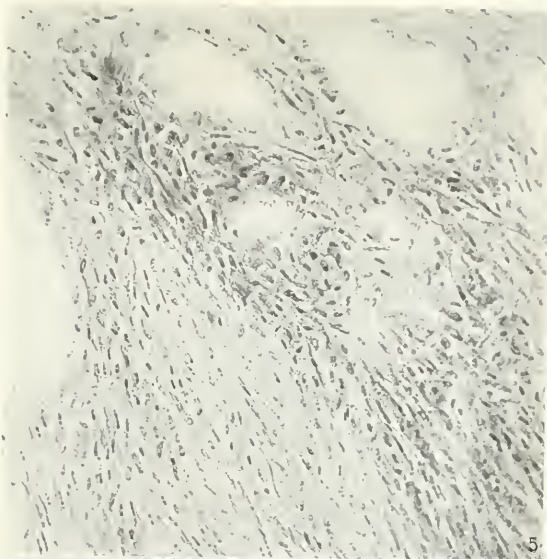
(Ebeling and Fischer: Fibroblasts and epithelial cells.)



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THE RELATION BETWEEN THE ACCUMULATION OF GLOBULINS AND THE APPEARANCE OF AGGLUTININS IN THE BLOOD OF NEW-BORN CALVES.

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Recent work has demonstrated with regard to the agglutinins of *Bacillus abortus* that the blood of the normal new-born calf before it has received colostrum does not contain agglutinins. If the calf receives colostrum from a cow with a high titer for *Bacillus abortus*, agglutinins will appear in the blood in a relatively short time (1). Furthermore, the blood of a new-born calf before it has suckled does not contain appreciable quantities of protein precipitated by concentrations of sodium sulfate which are less than 17.4 per cent whereas after it has ingested colostrum these proteins will be present in relatively large amounts (2). The ingestion of milk does not result in the increased appearance of the two globulin fractions of the blood. The data presented in this paper bear upon the relation which exists between the appearance of the agglutinins and the accumulation of globulins in the blood of new-born calves and upon the protein fractions in the colostrum and blood with which the agglutinins are associated.

I.

The Simultaneous Appearance of Agglutinins and Certain Globulins in the Blood of New-Born Calves.

Presentation of Data.—The method of collecting the samples of blood, and the determination of the agglutinin titer of blood and colostrum have already been presented (1, 3). The procedure for the determination of the proteins of blood and colostrum has also been

given (2, 4, 5). The accompanying tables contain representative data.

The data contained in Table I illustrate the rapidity with which the agglutinins and globulins are absorbed by the new-born calf. In the first case (calf of Cow 664), an agglutinin titer of 1:80 is obtained

TABLE I.

*Data Showing the Changes in the Agglutinin Titer and in the Proteins of the Blood of Calves Following the Ingestion of Colostrum.**

Age of calf.	Total nitrogen.	Euglobulin.	Pseudoglobulin I.	Pseudoglobulin II.	Total globulin.	Albumin.	Non-protein nitrogen.	Agglutination.
Calf of Cow 664.								
20 min.	0.776	0.018	0.072	0.184	0.273	0.430	0.073	—
Calf allowed to suckle dam.								
4 hrs.	0.896	0.098	0.218	0.115	0.431	0.391	0.073	1:80
6 " 10 min.	1.066	0.162	0.303	0.183	0.648	0.354	0.064	1:160
14 " 30 "	1.399	0.512	0.401	0.175	1.088	0.247	0.064	1:320
11 days.	1.352	0.333	0.469	0.149	0.951	0.367	0.034	
Calf of Cow 411.								
Dam's blood.	1.135	0.090	0.286	0.119	0.495	0.606	0.034	—
35 min.	0.759		0.068	0.154	0.222	0.460		
Calf allowed to suckle dam.†								
1 hr., 50 min.	0.700		0.073	0.149	0.222	0.422	0.077	1:40
3 hrs., 50 "	0.802	0.038	0.094	0.171	0.303	0.422		1:640
18 "	0.925	0.085	0.290	0.153	0.443	0.354	0.043	1:2,560

* Results in this and the accompanying tables are expressed as grams of nitrogen in 100 cc. of blood, and as the highest dilution at which agglutination was positive.

† The composition of the first colostrum was as follows, expressed as grams of nitrogen per 100 cc. of centrifuged colostrum: total nitrogen 2.747, euglobulin N 0.899, casein N 1.097, pseudoglobulin I N 0.03, pseudoglobulin II N 0.154, albumin N 0.224, non-protein N 0.074.

within less than 3 hours. In the same time the amount of pseudoglobulin I has increased from 0.072 gm. of nitrogen per 100 cc. of blood to 0.218 gm. of nitrogen and the euglobulin has increased in a like proportion; about 2 hours later analyses showed further increases in both the agglutinin titer and the globulin content of the serum.

In the second case (calf of Cow 411), the increase in the globulin content is not as rapid but the agglutinin titer is much higher. With both animals an absorption of agglutinins is accompanied by an increased amount of globulin in the blood.

The effect of withholding colostrum from calves for a short period of time is shown in the first portions of the data contained in Table II. From an inspection of the data it is evident that when colostrum is withheld from a calf and ordinary milk is given, neither the agglutinins nor the globulins appear in the blood to an appreciable extent for at least 21 hours. In this time a calf which has received colostrum soon after birth will have accumulated a considerable quantity of globulin in its blood and also agglutinins, if they are present.

When colostrum is fed at the end of 17 or 21 hours, in three cases¹ agglutinins appear in the blood and there is a very definite increase in the concentration of euglobulin and pseudoglobulin I (calf of Cow 3401, Table II) and of euglobulin and pseudoglobulin in the case of the calf of Cow 627.²

There is a difference between the calf of Cow 3401 and the calf of Cow 627 in that in the first case the calf received milk which had been refrigerated for approximately 17 hours and then warmed, and later another milking of colostrum was fed which had been held only 8 hours before feeding. The calf of Cow 627 suckled another cow (No. 626) immediately after bleeding at 21½ hours. The agglutinin titer of the blood in each of these cases reflects the titer of the colostrum which the calf ingested.

¹ A third case is not reproduced here but is to be found in a previous publication (2), Calf B. This calf received 36 ounces of colostrum, which had been refrigerated and warmed before it was fed, approximately 21 hours after birth, and later 92 ounces of colostrum at approximately 28½ hours after birth. The agglutinin titer of the blood serum was negative at birth and first gave a reaction at 28½ hours, 1:160; at 45 hours the titer was 1:160, and at 71 hours it was 1:320.

² At the time the blood of this calf was analyzed the presence of two pseudoglobulins had not been established for the analytical procedures used, and analyses were not made at 17.4 per cent of sodium sulfate. The data presented represent pseudoglobulins I and II. Analyses made later on these samples showed the presence of pseudoglobulin I following the ingestion of colostrum and the essential absence of this protein before receiving colostrum. The data are not given since they are definitely open to question.

TABLE II.

Data Showing the Changes in the Agglutinin Titer and in the Proteins of the Blood of Calves Following the Ingestion of Colostrum. Effect of Withholding Colostrum for a Number of Hours.

Age of calf.	Total nitrogen.	Eu-globulin.	Pseudo-globulin I.	Pseudo-globulin II.	Total globulin.	Albumin.	Non-protein nitrogen.	Agglutination.
Calf born of a low titer cow (No. 3401), restrained from taking colostrum until after two blood examinations, then fed colostrum which had been refrigerated and warmed. Titer of colostrum 1:160.								
1 hr., 20 min.	0.805	0.000	0.001	0.102	0.289	0.463	0.053	—
17 hrs., 20 "	0.693	0.076	0.000	0.153	0.229	0.416	0.048	—
Fed 96 ounces of colostrum which had been refrigerated and warmed.								
21 hrs., 50 min.	0.812	0.068	0.131	0.110	0.309	0.459	0.044	1:20
Fed colostrum at 1.30 p.m. taken from dam at 5.30 a.m.								
26 hrs.	1.046	0.131	0.228	0.149	0.508	0.481	0.057	1:40
44 "	0.954	0.144	0.298	0.048	0.490	0.394	0.070	1:40
68 "	0.854	0.097	0.245	0.039	0.381	0.407	0.066	1:20

Calf of Cow 627. Restrained from taking colostrum until after three examinations of blood, during which time it was fed milk free from agglutinins, then allowed to suckle a high titer cow (No. 626) which had given birth to a calf the same day. Titer of colostrum 1:2,560.

1 hr., 50 min.	0.652	0.053		0.120*	0.173	0.416	0.052	—
7 hrs., 10 "	0.598	0.047		0.175	0.222	0.328	0.048	—
21 " 30 "	0.638	0.034		0.173	0.207	0.378	0.053	—
Calf placed with Cow 626 immediately after bleeding.								
32 hrs.	0.648	0.136		0.170	0.306	0.302	0.035	1:640
46 " 50 min.	0.772	0.142		0.248	0.380	0.339	0.053	1:640
71 " 20 "	0.839	0.191		0.186	0.377	0.409	0.053	1:640
79 " 50 "	0.778	0.130		0.178	0.308	0.431	0.039	1:1,280
93 " 20 "	0.774	0.135		0.164	0.299	0.422	0.053	—

Calf born of a high titer cow (No. 634), restrained from taking colostrum until after three blood examinations, during which time it received milk free from agglutinins, then fed the dam's colostrum which had been refrigerated and warmed. Titer of colostrum before refrigeration 1:640.

1 hr.	0.740	0.035		0.252*	0.287	0.400	0.053	—
6 hrs.	0.774	0.021		0.271	0.292	0.438	0.044	—
22 " 30 min.	0.660	0.039		0.166	0.205	0.407	0.048	—
Calf received 33 ounces of colostrum immediately after bleeding.								
29 hrs.	0.791	0.126		0.197	0.233	0.420	0.048	—
45 "	0.772	0.096		0.140	0.236	0.483	0.053	—
53 "	0.843	0.077		0.200	0.277	0.523	0.053	—
3 days.	0.832	0.094		0.212	0.268	0.489	0.044	—
4 "	0.717	0.065		0.175	0.240	0.429	0.048	—
5 "	0.740	0.084		0.175	0.259	0.438	0.043	—
7 "	0.730	0.095		0.184	0.279	0.407	0.044	—

* The remaining data in this column refer to pseudoglobulin I plus pseudoglobulin II.

The third case in this series (calf of Cow 634) received colostrum which had been refrigerated for approximately 22 hours, and showed but a slight increase in the globulin content of the blood and no agglutinins. The remarks just made with regard to the analysis of the blood of the calf of Cow 627² apply to this case. The globulin content of the colostrum was not determined. It is impossible to say, therefore, whether the failure to obtain a marked rise in the globulin content of the blood serum was due to the low globulin content of the colostrum, to the effect of refrigeration, or to the age of the calf. The latter condition would seem to be ruled out by the results of the other two experiments and on Calf B previously reported (2). Subsequent data indicate that the composition of the colostrum does have a relation to the kind and quantity of globulin which appears in the blood.

DISCUSSION.

A definite relation between the absorption of globulins and the absorption of agglutinins by new-born calves is brought out in the data presented. The absorption of euglobulin and pseudoglobulin I³ is a process independent of the presence or absence of agglutinins for *Bacillus abortus*. Whether or not agglutinins can be obtained, and absorbed independent of the globulins we do not know, but it appears doubtful from the work of investigators who have studied the chemistry of immunity. We have fractioned samples of milk with a high agglutinin titer which did not contain a marked increase in globulin content, but the agglutinins were removed by concentrations of salt which remove the globulins.

From the data presented it is evident that the agglutinins which the new-born animal acquires by the ingestion of colostrum are associated with the direct absorption of the globulins of colostrum.

There are numerous references in the literature to the absorption of unchanged foreign protein based upon biological tests. With regard to the absorption of colostrum we have the work of Langer, Bauer, and Bauereisen. Langer (6)

³ By euglobulin is meant protein precipitated from serum or colostrum at 14.0 to 14.2 per cent of sodium sulfate, *i.e.* 14.2 gm. of sodium sulfate contained in 100 cc. of solution at 37°C., and by pseudoglobulin I is meant protein precipitated between 14.2 and 17.4 to 18.4 per cent of sodium sulfate and exclusive of casein in the case of colostrum.

demonstrated, by means of the precipitin reaction, the absence of substances in the blood of the new-born calf which would react with colostrum antiserum, whereas colostrum antiserum and cow blood did react. After suckling, and as early as 6 hours after suckling, substances appeared in the blood of the calf which reacted with colostrum antiserum. The maximum content of reacting substance was reached on the 2nd day, after which the values were relatively constant. A new-born calf allowed to suckle a cow which had given birth 3 weeks previously did not have in its blood, after 48 hours, substances which would react with colostrum antiserum. Langer concluded that the blood of the new-born received from colostrum certain additions by direct absorption which made the blood similar in composition to that of its mother.

Bauer (7) presented evidence for new-born infants, based upon the complement fixation test, similar to that of Langer's. He concludes, however, that since all children contain the acquired substance within the first 2 months no matter what the diet may be, that the acquired protein is formed and increases during early life. On the other hand, Bauer used antiserum to the soluble proteins of cow's milk and obtained reactions with the blood of the new-born calf which had not suckled. From this observation he concluded that a calf at birth is more advanced in its development than the infant.

Bauereisen (8) studied, with the precipitin reaction, the problem of the relation of blood of the new-born to the blood of the mother, to colostrum, and to milk. He conducted his experiments with reacting substances of equal protein content, since the blood of the new-born is low in protein, and likewise milk, as compared with colostrum. Under such conditions Bauereisen found the blood from the umbilical cord of calves to give identical reactions with the antisera of colostrum, milk protein, and casein. Antisera of blood obtained from the umbilical cord did not give a high titer. However, colostrum antiserum precipitated the blood from the umbilical cord of infants. He concludes, therefore, that the substances present in the blood of the mother are also present in the blood of the unsuckled new-born infant. The difficulties of the previous investigators had been in the difference in the protein content of the two bloods.

It is to be remembered that in the work just reviewed the investigators were working with mixtures of proteins, with the exception of casein, and some of the data suggest that even the casein was impure. Bauereisen was probably correct in his deductions from his experimental data, but from the results presented here and that of Howe (2) it is evident that Langer was correct in assuming that the new-born animal receives from colostrum certain additions to its blood. These additions are, as we have shown, globulin fractions, the fractions which carry agglutinins. There are other, quantitative, differences between the serum of the new-born and that of the adult, some of which we hope to present in the near future.

II.

The Protein Fractions of Colostrum and of Serum with Which the Agglutinins for Bacillus abortus Are Associated.

Presentation of Data.—Samples of colostrum and of serum with high agglutinin titers for *Bacillus abortus* were fractioned with sodium sulfate at 37°C. to determine the protein fraction or fractions which would contain the agglutinins. Proteins when precipitated are readily soluble in water and the adherent salt. The general procedure for the separation of protein fractions was to precipitate small quantities, 0.5 to 1.0 cc. of colostrum or serum, with various concentrations of sodium sulfate, wash the precipitate with the concentration of sodium sulfate used in precipitation, and then to dissolve the precipitated protein in a known quantity of distilled water. The final concentration of the protein was then known within approximate limits; the assumption was made that the quantity of water retained in the filter after dissolving the precipitate was the same as that which was present after filtration and washing. The protein solutions contained sodium sulfate in variable amounts which depended upon the concentration of sodium sulfate used. That this salt did not in itself cause agglutination nor inhibit agglutination at the high dilutions was demonstrated by control experiments in which the concentrations of sodium sulfate employed for precipitation were used and by tests upon filtrates from precipitations which completely removed the agglutinins. In some cases the proteins were reprecipitated and again tested for agglutinins. The procedure followed in testing for agglutinins has been described elsewhere (3). Representative data on serum and colostrum are contained in Tables III to IX.

The removal of agglutinins from serum by sodium sulfate precipitation is shown in Tables III and IV. The data in Table III relate to the agglutinin value of the protein precipitated from serum at various concentrations of sodium sulfate, and also of the original filtrates from such precipitations. One set of data is the complement of the other. It is evident that a considerable proportion of the agglutinins is removed by 14.5 per cent of sodium sulfate and that complete removal is accomplished at 16.4 per cent of sodium sulfate. Similar results are shown in Table IV. In this case, however, instead of

TABLE III.

The Agglutinin Titer of Protein Fractions from Serum, Separated with Sodium Sulfate at Definite Concentrations, and of the Filtrates from These Separations.

Fractions.	Dilutions.						Control.
	1:20	1:40	1:80	1:160	1:320	1:640	
Serum.	C.	C.	++ ++	++	+	=	-
<i>Na₂SO₄, per cent</i>							
13.5		++	+	=	-	-	-
14.2		++	++ +	+	-	-	-
14.5		++ ++	++ ++	++	+	-	-
15.5		++	++ +	++ +	+	-	-
16.4		++ +	++ +	++ +	++	-	-
17.4		++	++ +	++	++	-	-
21.5		++	++ ++	++	+	-	-
Filtrates.							
13.5 F		C.	C.	++ +	+	-	-
14.2 F		"	++ +	+	-	-	-
14.5 F		++ ++	++	-	-	-	-
15.5 F		++ ++	+	-	-	-	-
16.4 F		+	-	-	-	-	-
17.4 F		-	-	-	-	-	-
21.5 F		-	-	-	-	-	-

examining the filtrate from the original precipitation directly, the agglutinin titer of the protein precipitated at 18 per cent of sodium sulfate was employed; at this concentration all agglutinins have been

TABLE IV.

*The Agglutinin Titer of Protein Fractions from Serum Which Were Precipitated with Sodium Sulfate and of the Protein in the Filtrates Precipitated by Increasing the Concentration of Sodium Sulfate to 18 Per Cent.**

Fractions.	Dilutions.								Control.
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	
Serum.	++ ++	++ ++	++ +	++	++ +	++ +	++ +	++	—
<i>Na₂SO₄, per cent</i>									
13.5		++	++	+	±	—	—	—	—
14.5		++ +	++ +	++	+	±	—	—	—
15.5		++	++ +	++	++	±	—	—	—
17.4		++ +	++ +	++	++	—	—	—	—
Filtrate protein.									
13.5/18		++ +	++ ++	++	+	—	—	—	—
14.5/18		++ +	+	—	—	—	—	—	—
15.5/18		—	—	—	—	—	—	—	—

* The solutions of the precipitates produced by 14.5, 15.5, and 17.4 per cent of sodium sulfate contained a dark precipitate the following day which was removed by centrifugation.

removed in every case studied; in fact, 16.4 per cent of sodium sulfate is usually sufficient.

Data relating to the removal of agglutinins from colostrum are contained in Tables V to IX. The data in Table V illustrate the

TABLE V.

The Agglutinin Titer of Protein Fractions from Colostrum, Separated with Sodium Sulfate, and of the Filtrates from These Fractions.

Fractions.	Dilutions.										Control.
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	
Colostrum.	++ +	++ +	++ +	++ +	++ +	++ ++	++ ++	++ ++	++ +	+	-
<i>Na₂SO₄, per cent</i>											
7.5	++ +	+	-	-	-	-	-	-	-	-	-
9.5	++ ++	++ +	+	-	-	-	-	-	-	-	-
10.5	++ ++	++ +	+	-	-	-	-	-	-	-	-
12.5	++ ++	++ ++	++ ++	++ ++	++ ++	++ +	+	-	-	-	-
13.5	++ ++	++ ++	++ ++	++ ++	++ ++	++ +	++ +	+	-	-	-
14.2	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	++ +	++	±	-	-
14.5	++ +	++ +	++ +	++ +	++ ++	++ ++	++	+	-	-	-
15.5	++ +	++ +	++ +	++ ++	++ ++	++ ++	++ ++	++ +	+	-	-
16.4	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	++ +	++	+	-
17.4	++ +	++ +	++ +	++ +	++ ++	++ ++	++ ++	++ +	++	-	-
Filtrates.											
7.5 F	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	++ +	+	-	-	-
11.5 F	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	-	-	-	-	-

TABLE V—*Concluded.*

Fractions.	Dilutions.										Control.
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	
Filtrates.											
<i>Na₂SO₄, per cent</i>											
12.5 F	++	++	++	++	++	+	—	—	—	—	—
	++	++	++	++	++						
13.5 F	++	++	++	++	++	++	+	—	—	—	—
	++	++	++	++	++						
14.2 F	++	++	++	++	+	—	—	—	—	—	—
	++	++	++	++							
17.4 F	+	—	—	—	—	—	—	—	—	—	—

extent to which the agglutinins are removed by each concentration of sodium sulfate. The agglutinin titers of the precipitates increase up to approximately 16.4 per cent of sodium sulfate. A considerable proportion of the agglutinins is precipitated with the proteins separating at 12.5 per cent of sodium sulfate. The precipitates in these cases were obtained from 1 cc. of colostrum and were washed four times with sodium sulfate solutions of the concentrations used in precipitating them. The titrations of the filtrates from the original precipitations show that the agglutinins have been completely removed at 17.4 per cent of sodium sulfate, a concentration which precipitates all of the protein which we have assumed to be pseudoglobulin I. The demonstration of the complete removal of agglutinins by examination of the protein contained in the filtrates, such as was done for serum (Table IV), is contained in Table VII.

There is a possibility in the procedures followed in separating the protein fractions that the precipitates are contaminated by solution retained on the filter or adsorbed on the precipitate. The experiment detailed in Table VI was conducted in an attempt to obviate this possibility. After precipitating the proteins and washing the precipitates, they were dissolved in water and tested for agglutinins. An aliquot portion of the remaining solution was taken, the protein reprecipitated, and, after again washing thoroughly, the precipitate

TABLE VI.

*The Agglutinin Titer of (a) Protein Fractions Precipitated from Colostrum by Sodium Sulfate and (b) after Reprecipitation of the Same Fractions.**

Fractions.	Nitrogen per 100 cc.	Dilutions.									Control.
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	
	<i>gm.</i>										
Rennet whey (1).	3.439†	++	++	++	++	++	++	++			
			+	+	+	++	++	++			
" " (2).		++	++	++	++	++	++	++	++	++	—
		+	+	++	+	+	+	++	++		
<i>Na₂SO₄, per cent</i>											
13.8 (a)	0.742	++	C.	++	++	++	++				—
		++		++	++	+					
13.8 (b)		++	++	++	++	++	++	+	—	—	—
		++	++	++	++	+					
14.0 (a)	0.998	++	++	C.	++	++	++	++			—
		+	++		++	++	+				
14.0 (b)		++	++	++	++	++	++	++	—	—	—
		++	++	++	++	+					
14.2 (a)	1.006	++	++	++	++	++	++	++			—
					++	+	++				
14.2 (b)		++	++	++	++	++	++	++	++	+	—
		++	++	++	++	++	++	+			
18.4 (a)	2.380	++	++	++	++	++	++	++			—
		+	++	++	++	+	+				
18.4/14.2 (b)		++	++	++	++	++	++	++	+	—	—
		++	++	++	++	+	+				
Filtrate from 18.4/14.2		C.	++	+	—	—	—	—	—	—	—

* The first agglutination series and rennet whey (1) extended only to a dilution of 1:1,280. In the case of 18.4/14.2 (b) the dissolved protein precipitated at 18.4 per cent of sodium sulfate was reprecipitated at 14.2 per cent of sodium sulfate.

† Colostrum.

was dissolved and tested. It is evident that the agglutinins remained with the protein material.

TABLE VII.

Data Showing (1) the Effect of Clearing Colostrum with Sodium Oxalate, (2) Precipitation with Carbon Dioxide, and (3) the Agglutinins Remaining in Solution after Precipitation of Proteins at Given Percentages Which Are Afterward Precipitable by 18 Per Cent of Sodium Sulfate.

Fractions.	Nitrogen per 100 cc.	Dilutions.								Control
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	
	<i>gm.</i>									
Colostrum, whey.	2.640*	++	++	++	++	++	++	++	+	—
		+	++	++	++	+	+			
Colostrum, oxalated.		++	++	++	++	++	++	++	++	—
		++	++	++	++	++	++	+		
CO ₂ , oxalated.	1.365	++	++	++	++	++	++	+	±	—
			+	+	+	+				
CO ₂	1.365	++	++	++	++	++	++	+	+	—
		+			+	+				
CO ₂ , filtrate.	1.275		+	+	++	++	++	+	±	—
						++				
<i>Na₂SO₄, per cent</i>										
13.5/18		++	++	++	++	+	—	—	—	—
			+	++	++					
14.5/18		++	++	++	+	—	—	—	—	—
		++	++							
15.5/18		—	—	—	—	—	—	—	—	—
16.4/18		—	—	—	—	—	—	—	—	—

* Colostrum.

In the course of experiments relating to the determination of the proteins of colostrum, attempts were made to utilize carbon dioxide as an agent for the separation of the proteins. It was found that variable results were obtained, in some cases casein alone was precipitated and in others euglobulin and casein. In Table VII are data

relating to the agglutinin titer of the proteins separated by carbon dioxide, from which it appears that a large proportion of the agglutinins was removed by carbon dioxide. In this case the carbon dioxide removed both casein and euglobulin, a fact which is evident from other

TABLE VIII.

The Agglutinin Titer of Protein Fractions Removed Successively from Solution by Sodium Sulfate or by Acetic Acid.

Fractions.	Nitrogen per 100 cc.	Dilutions.							Control.
		1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	
	<i>gm.</i>								
Colostrum, oxalated.	3.872	++	++	++	++	++	++	+	—
		++	++	++	++	++	+		
Procedure a.									
Casein, direct precipitation.	0.648	—	—	—	—	—	—	—	—
Na ₂ SO ₄ 14.2 per cent after casein.	0.804	++	++	++	+	—	—	—	—
		+	+	++					
Na ₂ SO ₄ 17.4 per cent after 14.2 per cent.	1.201	++	++	++	++	++	+	—	—
			+	++	++	+			
Procedure b.									
Na ₂ SO ₄ 14.2 per cent.	0.993	++	++	++	++	++	++	—	—
			+	+	++	+			
Casein after 14.2 per cent.	0.755	—	—	—	—	—	—	—	—
Na ₂ SO ₄ 17.4 per cent after 14.2 per cent less casein.	1.126	++	++	++	++	+	—	—	—
		++	++	++	+				

analytical results obtained at the same time; hence the precipitation of agglutinins is to be compared with those obtained at 14.2 per cent of sodium sulfate. The agglutinin titer of protein not precipitated at 14.5 per cent of sodium sulfate (14.5/18) in the other experiments is to be compared with the data relating to the carbon dioxide precipitation.

Occasionally a sample of colostrum is not coagulated readily by

rennin, which interferes with the titration of the agglutinins. Sodium oxalate will readily remove the opacity of a solution of milk or colostrum, particularly if it be diluted with one or two volumes of a salt solution or water (5). Such additions of sodium oxalate have not interfered with the determination of agglutinins in a number of cases in our experiments. An example is contained in Table VII. On the other hand, the introduction of such a salt into the agglutinating system is not desirable. We have, therefore, used the whey unless it was impossible to do so.

The experiments outlined with regard to colostrum have involved the determination of the agglutinin titer of single proteins or mixtures of proteins precipitated in each case from a definite quantity of the original colostrum. In Table VIII are data which bear upon the removal of agglutinins by the successive precipitation of proteins or groups of proteins from one sample of colostrum. Two portions of the colostrum were used. From the dilute solution of one (*a*) the casein was removed by the addition of acetic acid and then, after careful neutralization, the globulins were precipitated with sodium sulfate. In the second portion (*b*) sufficient sodium sulfate was added to give the solution a concentration of 14.2 per cent, and after filtration the casein was precipitated with acetic acid, the filtrate neutralized, and sodium sulfate added to make a concentration of 17.4 per cent to separate the pseudoglobulin I fraction.

The data indicate that casein does not carry the agglutinins; the precipitated casein was dissolved by the careful addition of 0.1 N NaOH. The discrepancy between the agglutinin titers obtained at 14.2 per cent of sodium sulfate after casein has been removed and before the removal of casein is due, we believe, to the change in the precipitability of the globulins as the result of acidification and neutralization following the direct precipitation of casein from the original solutions of colostrum. That different quantities of protein are precipitated is evident from the different amounts of protein nitrogen present in these cases. This difficulty has been experienced in other experiments relating to agglutinins and in attempts to precipitate the proteins of colostrum (5). The variable results found at 14.2 per cent of sodium sulfate are reflected in the agglutinin titers of the subsequent fractions. The most important result is, however,

the failure to obtain agglutinins with the casein fractions. That the absence of agglutinins is not the result of destruction resulting from acidification and neutralization is evident from the demonstration of agglutinins in the subsequent fractions precipitated by sodium sulfate after neutralization.

TABLE IX.

The Agglutinin Titer of Protein Fractions of Milk.

Fractions.	Nitrogen per 100 cc.	Dilutions.							Control.
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	
	gm.								
Milk, whey.	0.478	C.	++ ++	++ ++	++ +	++	+	-	-
<i>Na₂SO₄, per cent</i>									
13.5	0.022	++ ++	++ +	++	+	-	-	-	-
14.0 ⁹	0.017	++ ++	++ ++	++	+	-	-	-	-
14.2	0.034	++ ++	++ +	++	±	-	-	-	-
17.4	0.277	++ ++	++ ++	++ +	++ +	++	±	-	-

Certain samples of milk having a relatively high agglutinin titer were available. Fractionation of such samples (Table IX) gave results similar to those found for colostrum. On the other hand, if we exclude the casein the agglutinins are associated with a relatively small amount of protein.

DISCUSSION.

The data presented with regard to the precipitation of agglutinins for *Bacillus abortus* in blood and colostrum indicate definitely that the protein, or protein mixtures, precipitated up to and including 16.4 per cent of sodium sulfate carries with it the agglutinins. It is this fraction, which is absent from the blood of the new-born calf, which is abundant in most samples of colostrum and which is absorbed directly

by the new-born animal. The association of agglutinins with a particular protein fraction is not clear-cut. It appears that a large proportion of the agglutinins is associated with the fraction which is precipitated by 14.2 per cent of sodium sulfate, euglobulin. On the other hand, agglutinins were found in later fractions, 14.2 to 16.4 per cent of sodium sulfate. If we assume that the agglutinins are associated only with the euglobulin fraction, precipitated by 14.2 per cent of sodium sulfate or by carbon dioxide, then it is necessary to conclude that the agglutinins appearing in the later fractions are there because of a failure to precipitate all of the euglobulins in the process of fractionation. The method of separation does not preclude such a possibility. Certain results obtained in testing samples of colostrum for agglutinins, in our search for a high titer colostrum, leave the situation at present just where a literal interpretation of the data given would place it; *i.e.*, that the agglutinins are associated with the protein fractions which are precipitated up to and including 16.4 per cent of sodium sulfate. The observations referred to related to samples of colostrum having a low titer with a euglobulin content which was high or relatively high. In these cases the agglutinins did not appear, or but a trace was present, in the fraction precipitated at 14.0 to 14.2 per cent of sodium sulfate, but did appear in fractions precipitated at 18.4 per cent of sodium sulfate; in these preliminary tests only two concentrations of sodium sulfate were used. It is evident, however, that while there may be difficulties of separation they concern only the euglobulin fraction and the pseudoglobulin I fraction and not the protein precipitated at higher concentrations of sodium sulfate.

It is not possible to draw any more definite conclusions through a consideration of past work. Gibson and Collins (9) fractioned immune sera of various kinds from different animals and were unable to show that the agglutinins or anti-toxins were associated exclusively with any particular fraction. They did find that a large proportion of a particular antibody was found either with the euglobulin fraction or with the pseudoglobulin fraction. These conclusions were reached after consideration of the literature in which immune bodies were held to be associated with definite fractions.

Hartley (10) has since held that the immune bodies against rinderpest were associated with the euglobulin fractions of blood serum. He also found an increase in the euglobulin fraction as the result of immunization. Homer (11) has

studied the fractionation of diphtheria antitoxin serum with sodium sulfate as well as with ammonium sulfate. With regard to the distribution of the antitoxin her work was similar to that of Gibson and Collins in that, by fractioning with different concentrations of ammonium or sodium sulfate, some of the antitoxin was found in the euglobulin-pseudoglobulin zone, but most of the antitoxin was found with the pseudoglobulin. It was further shown that "the percentage precipitation of the antitoxin with the proteins precipitated at various concentrations of sodium sulphate is a linear measure of the percentage precipitation of the antitoxin-bearing proteins." This relationship is undisturbed by heat denaturation of plasma adjusted to a definite hydron concentration. There is a particular difference between our results and those of other investigators in that the proteins were precipitated at a relatively high dilution, a condition which should tend to minimize the effect of contamination by adsorption.

SUMMARY.

A comparison of the appearance of the agglutinins for *Bacillus abortus* in the blood of new-born calves with the first appearance of globulins in the same blood following the ingestion of colostrum indicates that the agglutinins are associated with the globulins. These observations are supported by the removal of the agglutinins from serum or colostrum with concentrations of sodium sulfate which precipitate globulins present in the blood of calves which have ingested colostrum, but which are not present at birth.

Neither the association of immune bodies with globulins nor the direct absorption of protein by new-born animals is a new fact. The evidence presented is of particular value, however, in associating the appearance of certain protein fractions in the blood of the new-born animal with the simultaneous absorption of agglutinins.

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RABBIT SEPTICEMIA BACILLUS, TYPES D AND G, IN NORMAL RABBITS.

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INTRODUCTION.

In preparation for experiments on the epidemiology of rabbit septicemia, it was necessary to obtain rabbits which were free from infection with this organism. The normal rabbit stock of the laboratory consequently was examined by culturing of nasal mucous membranes. During the routine of this examination the following interesting observations were made.

EXPERIMENTAL.

Procedure for Detection of Type D.—A small sterile swab was carefully introduced into the nostril of the rabbit and subsequently streaked at once on 10 per cent horse serum agar plates, pH 7.4. The plates were incubated at 37°C. for 24 hours and examined.

Of the first series of twenty-nine animals examined, fifteen were found to harbor typical D organisms. These animals were healthy to all outward appearance. They were well nourished, of good appetite, and had no visible discharge from the nose. The Type D colonies were present in great abundance. In many of the rabbits they constituted 50 per cent or more of the nasal flora. The Type D are easily differentiated from other colonies by their high fluorescence in artificial light, their rather small size, smoothness of contour, and by the fact that they are very "soft" and tend to disintegrate completely when gently touched with a platinum wire. Other fluorescent colonies can either be shoved about on the agar surface, retaining their form perfectly, or break up into two or three distinct fragments.

Suspected Type D colonies are examined by hanging drop, oil immersion objective, and if found to yield non-motile, minute coccoid

rods, are subcultured to rabbit serum broth. These cultures are then examined for turbidity, bipolarity in Löffler's methylene blue stain, acid agglutination optimum, and agglutination by specific anti-D serum.

Type G in Normal Rabbits.

In the same series of twenty-nine animals, four nasal swabs yielded plates heavily seeded with colonies exactly resembling those of the mutant G form.¹ These colonies were translucent and of faintly bluish tinge, exhibited no fluorescence with artificial light, and had slightly irregular borders.

They were fished into serum broth and yielded the granular sedimenting growth characteristic of Microbe G, rabbit septicemia bacillus. The organisms were minute, non-motile, coccoid bacilli, growing singly or in pairs. In hanging drop, fresh preparation, they exhibited the typical shadows at the poles. Löffler's methylene blue demonstrated their bipolarity.

TABLE I.
Acid Agglutination Optimum of Suspected Type G.

pH.....	6.6	6.3	6.0	5.5	5.3	5.0	4.6	3.9	3.3	3.0
Result.	Tr.	+	++	++	++	++	C.	C.	+	Tr.

These results justified more minute examination, since it would be important to find out whether Type G, up till now observed only as a mutant from Type D, under cultural conditions, occurred naturally in the animal body.

Experiment 1. Acid Agglutination Optimum of Suspected Type G.—Organisms from a suspected Type G colony were planted in serum broth, and the 16 hour granular culture that resulted was washed four times and finally suspended in distilled water. 1 cc. quantities of this suspension were then mixed with 1 cc. of buffer solution of different hydrogen ion concentrations. The buffer solution used was glycoll-Na acetate- Na_2HPO_4 , $\frac{M}{12}$ concentration. Incubation 43°C . for 14 hours. The result is recorded in Table I.

The broad zone of hydrogen ion concentration over which agglutination took place is characteristic of Type G recovered from the

¹ De Kruif, P. H., *J. Exp. Med.*, 1922, xxxv, 561.

animal body in artificial infections.² The agglutination reaction by specific serum was next attempted.

Experiment 2. Agglutination of Suspected Type G by Rabbit Serum > D.—It has been observed in a preceding paper³ that Types D and G show antigenic community, and that inoculation with Type D results in a slightly stronger agglutinin response than that following injection of Type G. Rabbit serum anti-D was therefore used in this experiment. Observation of Table I shows that marked agglutination of the suspected organism occurs at pH 6.0. This fact makes it impossible to perform the agglutination reaction with 0.85 per cent NaCl dilutions of serum, since these would cause agglutination of the organism in dilutions no longer protected by the buffer action of the serum. It is apparent that, in a series of dilutions of serum with 0.85 per cent NaCl, the pH would vary from 7.4 in the concentrated, to 5.8–6.0 in the high dilutions.

TABLE II.
Agglutination of Suspected Type G by Anti-D Immune Serum.

Serum.	Agglutination.									
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Control. No serum.
Rabbit serum, anti-D.....	C.	C.	C.	++	—	—	—	—	—	—
Normal rabbit serum.....	++	+	+	—	—	—	—	—	—	—

The immune serum for this test was therefore diluted in glycocoll-Na acetate, Na_2HPO_4 buffer, pH 7.1. This insured the occurrence of a hydrogen ion concentration at which suspensions of the suspected organism were stable. The results of the agglutination test are given in Table II. Incubation, as usual, at 43°C. for 14 hours.

This experiment and other similar ones definitely identify the suspected organism as a member of the rabbit septicemia group. It remained to test its virulence on intrapleural injection into rabbits.

Experiment 3. Lack of Virulence of Suspected Type G.—A 6 hour serum broth culture of the suspected organism was carefully shaken up to secure uniform distribution of clumps, diluted to 5×10^{-2} cc. in broth. Amounts equivalent to 0.05 and 0.1 cc. of the original culture were inoculated intrapleurally into young rabbits of 600 gm. weight. No untoward result was discovered, although the animals were kept under observation for more than 30 days. This lack of virulence confirms the suspicion of the identity of this organism with the mutant G forms.

² De Kruij, P. H., *J. Gen. Physiol.*, 1922, iv, 387.

³ De Kruij, P. H., *J. Exp. Med.*, 1921, xxxiii, 773.

Subsequent examination of supposedly normal adult rabbits has revealed a fair proportion as carriers of this organism, which conforms in every characteristic to the G type arising from Type D by mutation in culture.

Experiment 4. Virulence of Type D Isolated from Nasal Mucous Membranes of Normal Rabbits.—It has been remarked in a preceding paragraph that fifteen out of twenty-nine rabbits of the normal stock were found to harbor typical Microbe D. It was considered of interest to test the virulence of these organisms, since that of strains arising from fatal bronchopneumonias is invariably high.^{1,3}

A Type D culture obtained from Rabbit 1 was accordingly seeded into 10 per cent rabbit serum broth and the virulence of the resulting 6 hour culture tested by intrapleural injection into young rabbits of 600 gm. weight. The technique of the test was identical with that described in a preceding paper.³ The result is recorded in Table III.

TABLE III.

Virulence of Type D Isolated from Mucous Membranes of Apparently Healthy Rabbits.

Rabbit No.	Weight of rabbit. <i>gm.</i>	Age of culture. <i>hrs.</i>	Amount injected intra-pleurally. <i>cc.</i>	Result.	Necropsy findings.
2	605	6	1×10^{-6}	Died in 36 hrs.	Typical fibrinopurulent pleuritis, pericarditis, and bronchopneumonia.
3	610	6	1×10^{-6}	" " 48 "	" "
4	610	6	1×10^{-4}	" " 54 "	" "
5	600	6	1×10^{-3}	" " 36 "	" "

This experiment was made on November 22, 1921. At the present date, March 31, 1922, Rabbit 1 from which this highly virulent Type D culture was isolated, is alive, of full weight, and good appetite, and still harbors Type D organisms. Similar results have been obtained with Type D isolated from other normal rabbits.

This observation is of fundamental importance in the epidemiological study of the bronchopneumonia of rabbits caused by the rabbit septicemia bacillus. It is clear that these animals may harbor on the nasal mucous membrane organisms which display a virulence, by intrapleural test, equal to that of strains recovered from fatal infections. It would appear, from this and other considerations to be revealed in later communications, that there is no strict relation-

ship between intrapleural virulence and invasibility occurring in natural infections. In a word, some resistance-lowering factor must be present to enable the virulent organism to produce the fatal bronchopneumonia.

Agglutinins in the Blood of Rabbits Naturally Infected with Types D and G.

Since it is possible for rabbits to harbor highly virulent Type D organisms on the nasal mucous membrane, and remain for a long time without signs of disease, it was considered important to discover whether the blood showed any evidence of immune substances against the rabbit septicemia bacillus.

TABLE IV.

Relation between Agglutination Titer and Presence of Types D and G on the Nasal Mucous Membrane.

14 hours at 43°C.; pH 7.1.

Rabbit No.	Examination of nasal mucous membrane.	Agglutination.							Titer of serum.†
		Dilution of serum + Type G at pH 7.1.							
		1:10*	1:20	1:40	1:80	1:160	1:320	Control.	
6	No Type D or G present.	+	Tr.	Tr.	—	—	—	—	< 1:10
7	“ “ “ “ “ “	+	—	—	—	—	—	—	< 1:10
8	“ “ “ “ “ “	++	+	Tr.	—	—	—	—	< 1:10
9	“ “ “ “ “ “	++	+	—	—	—	—	—	< 1:10
10	“ “ “ “ “ “	+	Tr.	Tr.	Tr.	Tr.	—	—	< 1:10
11	“ “ “ “ “ “	+	“	“	—	—	—	—	< 1:10
12	“ “ “ “ “ “	++	++	++	+	+	Tr.	—	< 1:10
13	Type D present.	C.	C.	++	Tr.	—	—	—	1:20
4	“ G “	“	“	C.	C.	+	—	—	1:80
14	No Type D or G present.	Tr.	Tr.	—	—	—	—	—	< 1:10
15	Type D present.	C.	C.	C.	++	+	—	—	1:40
16	No Type D or G present.	Tr.	—	—	—	—	—	—	< 1:10
17	“ “ “ “ “ “	+	Tr.	—	—	—	—	—	< 1:10
18	Type D present.	++	++	++	++	Tr.	—	—	< 1:10

* Represents dilution of serum only. Multiply by 2 to obtain the final dilution of serum + suspension.

† The titer of the serum is read as the smallest amount causing complete agglutination of Microbe G.

Experiment 5. Relation between Agglutination Titer and Presence of Types D and G on the Nasal Mucous Membrane.—The method chosen was that described in Experiment 2. Four times washed Type G, suspended in distilled water, was tested against the suspected serum, diluted with buffer (glycocoll-Na acetate- Na_2HPO_4), pH 7.1. This method was chosen because, first, Type G is far more sensitive to rabbit septicemia agglutinins than is Type D; second, because at this pH, Type G shows itself to be perfectly stable and amenable therefore to agglutination test.

TABLE V.

Relation between Agglutination Titer and Presence of Types D and G on the Nasal Mucous Membrane.

Rabbit No.	Examination of mucous membrane.	Titer of serum.
19	No Type D or G present.	< 1:10
20	" " " " " "	< 1:10
21	" " " " " "	< 1:10
22	" " " " " "	< 1:10
23	" " " " " "	< 1:10
24	" " " " " "	< 1:10
25	" " " " " "	< 1:10
26	Type D present.	1:80
27	" " " "	1:40
28	No Type D or G present.	< 1:10
29	Type G present.	1:80
30	" D "	1:40
2	No Type D or G present.	< 1:10
31	" " " " " "	< 1:10
32	" " " " " "	< 1:10
33	" " " " " "	< 1:10
34	" " " " " "	< 1:10
35	" " " " " "	< 1:10
36	Type D present.*	< 1:10

* Type D had been present for only 4 days. The animal was demonstrated to be D- and G-free for at least 2 months previous to the infection, which occurred by direct contact.

The blood was drawn from the hearts of the animals under test. The serum was diluted 1:10 in the above named buffer and then by 2's to 1:320. 1 cc. of each dilution was mixed with 1 cc. of Type G suspension—incubation at 43°C. for 14 hours. The results are summarized in Tables IV and V.

Further experiments of a similar nature are summarized in Table V. In all cases the finding "Type D or G not present" was entered

after three successive streakings on serum agar plates of mucous membrane swabs, made at 5 day intervals.

The experiments summarized in Tables IV and V show that the presence of Type D or G, bacillus of rabbit septicemia, on the nasal mucous membranes of rabbits is accompanied by distinct evidence of specific agglutinins in the blood.

Excepting in one case (Rabbit 1) complete agglutination of Type G always occurred in 1:20 or higher dilutions when Type D or G could be demonstrated on the nasal mucous membrane. On the other hand, *no* serum caused complete agglutination in 1:10 or higher when three successive streakings on serum agar plates had demonstrated the absence of Types D and G.

DISCUSSION.

These findings are important in that they demonstrate (1) that the carrier condition is associated with evidence of immunity, as determined by the presence of definite amounts of agglutinins in the blood; (2) that the serum of any rabbit, taken at random from a normal stock, is *not* to be considered normal so far as the rabbit septicemia bacillus is concerned.

SUMMARY.

Microbe G, rabbit septicemia bacillus, hitherto found only as a mutant in cultures of the rabbit septicemia bacillus, Type D, has been demonstrated to exist on the nasal mucous membranes of normal rabbits. This organism corresponds in lack of virulence, character of growth, acid agglutination optimum, and immune agglutination reaction, to the mutant G form described in previous papers.

Microbe D has been found to be present on the nasal mucous membranes of normal rabbits. These animals have survived for months with no evidence of infection other than the presence of the organism. These Type D organisms, despite their failure to cause fatal damage in their own host, are shown to possess the typical high virulence characteristic of this type, when injected intrapleurally into young rabbits.

Rabbits which are carriers of Type D or G possess a definite amount of immune agglutinins, as evidenced by test of their serum against Microbe G at pH 7.1.

Rabbits free from infection with these organisms invariably have yielded a serum which fails to agglutinate Type G completely in 1:10 or higher dilutions.

THE SOURCE OF THE MICROORGANISMS IN THE LUNGS OF NORMAL ANIMALS.

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Considerable discussion has arisen at various times relative to the sterility of the normal lung during life. Hildebrandt (1) claimed that inspired bacteria under normal conditions do not reach the lung but are deposited in the nasal passages. Babes (2) stated that the lungs of normal men and animals were sterile. The findings of Barthel (3) were similar. Beco (4) found organisms as far as the middle of the trachea in domestic animals but believed the lungs as a rule were sterile. On the other hand, the assertions that the lungs contain organisms are upheld by many. Neisser (5) in studying sections of the lungs of normal guinea pigs, rabbits, and mice encountered organisms in three instances. Dürck (6) found bacteria in the sixteen human lungs examined. Boni (7) succeeded in cultivating organisms from the lungs of fourteen swine. In eleven instances a pneumococcus of low virulence was obtained. From two of ten guinea pigs he obtained positive cultures. Nenninger (8) states that the lung cannot be considered as a germ-free organ since it is constantly invaded by organisms of the atmosphere and those from the upper respiratory passages. Thomson and Hewlett (9) calculated that at least 1,500 organisms were taken into the nasal passages per hour in London. They claim that the ciliary action of the lining epithelium soon rids the inspired air of microorganisms. They maintain that the lungs are sterile. An instance cited by them is an observation by Lister of a case of rib fracture in which the lung had been punctured and blood mixed with inspired air entered the pleural cavity. The blood did not undergo "decomposition." Lister pointed out that the particles filtered out before reaching the alveoli. In many instances the exact methods of cultivation have not been given by various observers.

Quensel (10) was able to show that the lungs were not bacteria-free. His material was obtained from the larger domestic animals immediately after slaughter at the abattoir. Pieces of normal lung were removed from the carcass as quickly as possible and transported to the laboratory in sterile glass containers. The surface of the tissue was then seared and bits removed with sterile instruments and crushed between sterile glass slides. The crushed material was used to inoculate tubes of agar and bouillon. Cultures were made from the lungs of

16 calves, 15 sheep, and 5 horses. From the calves, 23 of 62 tubes showed growth. Organisms were found in 19 of the 54 tubes from the sheep. The number of the positive tubes from the horses was relatively higher, 11 of 19 revealed organisms. Streptothrix, *B. subtilis*, and molds made up 64 per cent of the organisms cultivated. Streptothrix were most frequently obtained. Quensel in discussing his findings pointed to several possible sources of entrance. He suggested that the organisms may have entered the lungs with the passive flow of contaminated fluids (blood, stomach contents, etc.) during slaughter, or that they may have been carried downward during the last violent respirations. Air-borne contamination during the manipulations of the tissues was also considered as a source of error. He was inclined, however, to regard these organisms in most instances as legitimate inhabitants of the lungs. In sharp contrast to these findings is the statement that he found the lungs of guinea pigs free from bacteria.

Quensel was able in a few instances to cultivate streptothrix and other organisms from the bronchial lymph glands of the calf, horse, sheep, and pig.

It was known that the larger herbivora harbored organisms, but some doubt existed as to the presence of bacteria in the lungs of the laboratory animals. Their possible source and significance seemed of particular importance. With these points in view a series of observations and experiments was undertaken.

Cultural Studies from the Lungs of Animals.

The animals employed were apparently in good health. The smaller animals—such as rabbits, guinea pigs, mice, etc.—were chloroformed. It is customary to kill calves by stunning with a heavy blow on the head, the trachea is quickly clamped, and the large neck vessels severed. The same methods were employed in certain instances in the smaller animals but it was found that the method of killing did not materially alter the findings in the case of the rabbit or guinea pig.

The hair over the chest of the laboratory animals was shaved and the skin cleansed with 5 per cent phenol followed by alcohol. The skin was reflected and the underlying musculature seared. A triangular window of ribs and sternum was removed with sterile instruments. Small pieces cut from the borders, furthest removed from the trachea, of the various lobes were added to tubes of media. In calves large pieces from the borders of two lobes were removed with sterile instruments through incisions in the chest walls. From the

external edges of these small pieces were used for cultivations. Relatively simple media, such as slanted plain agar and agar to which a few drops of defibrinated horse blood had been added and veal infusion bouillon were employed. The bits of tissue were pushed

TABLE I.

Results of the Bacteriological Examinations of the Lungs of Normal Animals.

Animal.	No. examined.	No. of tubes inoculated.	No. of tubes showing growth.	Kinds of organisms.	
Calves.	9	74	63	Streptothrix,	45.
				<i>B. subtilis</i> ,	6.
				Molds,	8.
				Cocci,	2.
				Mixed (<i>B. subtilis</i> and streptothrix),	2.
Rabbits.	7	50	42	Streptothrix,	5.
				<i>B. subtilis</i> ,	20.
				Molds,	7.
				Cocci,	7.
				Mixed (<i>B. subtilis</i> , streptothrix, and mold),	3.
Guinea pigs.	12	72	54	Streptothrix,	39.
				<i>B. subtilis</i> ,	10.
				Molds,	3.
				Cocci,	2.
White mice.	8	36	11	Streptothrix,	5.
				<i>B. subtilis</i> ,	3.
				Cocci,	3.
" rats.	6	36	13	Streptothrix,	1.
				<i>B. subtilis</i> ,	2.
				Cocci,	2.
				Molds,	8.

down over the surface of the slants into the condensation fluid. All tubes were sealed with sealing wax. Tubes were incubated for 14 days at 38°C.

The results have been recorded in Table I.

The results recorded in Table I are striking. Among the herbivorous animals the proportion of tubes showing growth is consistently high, varying from 75 per cent in the case of the guinea pig to 85 per cent in the instance of the calf. In not a single instance did all the tubes inoculated from the lung remain sterile. The proportion of positive tubes from the mouse and rat is much lower, 31 and 38 per cent respectively.

It is probable that two of the sources suggested by Quensel for the presence of organisms in the lungs have been ruled out. The relatively simple technique precludes contamination to any great degree through manipulation of the tissues. That fluids such as blood and regurgitated material cannot enter the clamped trachea is obvious. Regurgitation of stomach contents has not occurred in the smaller animals killed with chloroform.

The following facts tend to show that the organisms are within the lung. If tubes containing bits of lung are examined at frequent intervals, the filaments or tufts of streptothrix are frequently seen growing out of the borders of the tissue mass. This has been observed repeatedly on both the pleural and cut borders. The rapid growths of *Bacillus subtilis* and molds begin from the water of condensation and extend upward.

Quensel's third contention, however, that organisms may have gained access during the last deep respirations is doubtless true. Should this be admitted, then it must be assumed that at many of the deep inspirations during life organisms may reach the lungs.

A striking feature is the great similarity of the type of organisms encountered in the various species, particularly in the herbivorous animals. Streptothrix appeared 87 times, *Bacillus subtilis* 41, molds 25. Practically all the organisms met with are spore bearers. It is well known that they are particularly abundant in hay and straw. It seemed probable that they originated in the hay and straw and were taken into the respiratory tract. This would account for the larger proportion cultivated from the herbivorous animals and explain their small numbers in the mouse and rat.

EXPERIMENTAL.

To establish this hypothesis a series of experiments was undertaken.

Experiment 1.—Full grown healthy rabbits were placed in sterile individual cages in a separate unit and fed the usual ration of oats, hay, and mangels. All food was washed thoroughly in running water and fed while moist. Newspapers were used for litter. All litter and uneaten food were removed daily. The control rabbits received the same ration unwashed and fed dry. After periods of 11 days to 2 weeks the animals were chloroformed and cultures prepared from the borders of the lungs. Bits of tissue the same size were used in cultivating from both groups of rabbits. The results are given in Table II.

TABLE II.

Influence of Moistened and Dry Food on the Number of Organisms Found in the Lung.

Animal.	No. examined.	No. of tubes inoculated.	No. of tubes showing growth.	Kinds of organisms.	
Experimental rabbits (fed washed hay and oats).	7	48	14 (29 per cent).	Streptothrix,	7.
				<i>B. subtilis</i> ,	7.
Control rabbits (fed in usual manner).	6	44	40 (91 per cent).	Streptothrix,	11
				<i>B. subtilis</i> ,	22.
				Molds,	1.
				Mixed (streptothrix, <i>B. subtilis</i> , and molds),	6.

The results recorded in Table II are significant. The actual decrease in the number of tubes containing growth is striking. The inference that forms usually occurring in hay and straw may reach the lung is well founded. Similar experiments were tried on the guinea pig. A diminution always occurred in the number of tubes containing organisms but the results were never so striking.

By modifying the experiment somewhat the results obtained were very much the same.

Experiment 2.—Guinea pigs of the same age were divided into three lots. The cages and the litter were similar for each lot. All were kept in the same general atmosphere. One lot was fed on cabbage and oats, another lot was fed on grass and oats. The other controls received hay and oats with a little grass. After a

period of 1 month all the animals were killed and cultures prepared from the lungs. Since the results from the cabbage-fed and grass-fed animals were the same, they have been grouped together in Table III.

Much the same result was obtained in the case of the guinea pigs as in the rabbits under practically dust-free conditions. The diminution in the number of tubes showing growth from those kept in a reasonably clean atmosphere by withholding dry food matter is quite

TABLE III.

Results of Cultivations from the Lungs of Guinea Pigs Fed Grass and Hay.

Animal.	No. examined.	No. of tubes inoculated.	No. of tubes showing growth.	Kinds of organisms.	
Guinea pigs (grass or cabbage fed in place of hay).	7	38	10 (26 per cent).	Streptothrix,	8.
				<i>B. subtilis</i> ,	1.
				Molds,	1.
Control guinea pigs (ration the same except hay was fed).	7	37	33 (89 per cent).	Streptothrix,	22.
				<i>B. subtilis</i> ,	7.
				Molds,	4.

remarkable. The few organisms encountered in the experimental lots probably originated in the general atmosphere. In species thickly covered with hair there is no reason to suppose that spores may not exist for long periods. Methods to control this factor were not employed.

It is interesting to compare the results of the lung cultivations of the mice and rats recorded in Table I with those given in Tables II and III for rabbits and guinea pigs whose environments in general paralleled those of the rats and mice. The proportion of tubes showing growth is comparable. The rats and mice examined were kept in the same room with the other experimental animals. The same litter was used. The ration consisted of dog biscuit and whole corn. Hay or straw was not used in the cages. It seemed possible to account for the smaller number of organisms by the absence of materials heavily contaminated with spores. There was a possibility of some differences in anatomical structure of the upper respiratory tract which might not permit the free passage of many spores.

Experiment 3.—To test these points, twelve mice were divided into three groups. Each group was placed in a glass jar and received the same food. Lot 1 was given sawdust for litter and fed the usual ration of dog biscuit and corn. In the case of Lot 2 a mixture of finely cut straw was used for litter, the ration was the same as for Lot 1. With Lot 3 the litter consisted of finely cut newspaper which was changed daily. Both the corn and dog biscuit were sterilized in the autoclave for 20 minutes. After 8 days the mice were chloroformed and examined. The results are recorded in Table IV.

As a result of this experiment the fact that the flora of the lungs of the mouse can be influenced markedly by variations in the immediate

TABLE IV.

Proportion of Organisms Obtained from the Lungs of White Mice Kept under Ordinary, Dusty, and Dust-Free Conditions.

Lot 1. Ordinary conditions.		Lot 2. Straw as litter.		Lot 3. Dust-free conditions.	
Mice,	4.	Mice,	4.	Mice,	4.
Tubes inoculated,	20.	Tubes inoculated,	20.	Tubes inoculated,	20.
“ showing growth,	9.	“ showing growth,	16.	“ showing growth,	1.
“ remaining sterile,	11.	“ remaining sterile,	4.	“ remaining sterile,	19.
Tubes positive, <i>per cent</i> ,	45.	Tubes positive, <i>per cent</i> ,	80.	Tubes positive, <i>per cent</i> ,	5.
Growth obtained from the lungs of all mice.		Growth obtained from the lungs of all mice.		No growth obtained from the lungs of three mice.	

environment is brought out. Mice under ordinary conditions (Lot 1) show a moderate number of organisms within the lungs. The number can be increased until they approximate those found in the guinea pig or rabbit under normal conditions by subjecting them to the usual environment of the latter species. Evidently anatomical conditions are not responsible for the relatively few organisms encountered under usual methods of life. In Lot 3, in which the air was relatively free from dust and the spores of many forms, probably few organisms reached the lungs.

The experiments all tended to point to the immediate environment as the source of the organisms cultivated from the lungs. By withholding or supplying food and litter substances, such as hay and

straw, it has been possible to influence markedly the number of spore-bearing organisms cultivated. If it were possible to show that the streptothrix isolated from the lung resembled those found in the dust from hay, the chain of evidence would be complete. It has been stated that streptothrix are present in the greatest number of cultures. It was determined to compare the common lung strains with those obtained by exposing sterile plates to dust from hay and straw.

TABLE V.

Characters of the Streptothrix Obtained from the Lungs of Various Animals and Plates Exposed to the Dust of Hay and Straw.

Source and No. of strains.	Gram's stain.	Hemolysis in horse blood agar plate culture.	Milk.	Fioal hydrogen ion concentration in dextrose bouillon. <i>pH</i>	Gelatin.
Lungs of guinea pigs, 26.	+	+	Coagulated, alkaline, curd peptonized.	7.6-8.6	Liquefied.
" " rabbits, 4.					
" " calves, 6.					
" " mice, 5.					
Plates exposed to hay and straw, 5.	+	—	" "	7.6-8.6	"
Lungs of guinea pigs, 3.					
" " calves, 4.					
" " mice, 4.					
Plates exposed to hay and straw, 2.					

All cultures were Gram-positive filaments with true branches. The surface growth always became chalky as the media dried and the characteristic fragmentation of the filaments with the production of arthrospores was observed. Most of the cultures had a characteristic penetrating musty odor. Their biochemic activities are given in Table V.

The general characters of the streptothrix from the lungs of the guinea pig, rabbit, and calf resemble those obtained from hay and

straw. All liquefy gelatin, coagulate milk, and peptonize the curd. Alkali is produced in dextrose broth and milk. Some hemolyze horse blood in agar plate cultures, others fail to do so. The predominating color on potato is yellow, yellowish white, or orange. The rapidity with which gelatin and casein are digested varies in different cultures. Unfortunately, these are the characters given for most of the streptothrix but radical departures from these types have not been met with so that one feels safe in considering these organisms of the same group.

Quensel was able to cultivate streptothrix and *Bacillus subtilis* from the bronchial lymph glands of various animals in a few instances.

TABLE VI.

Results of Inoculations from the Bronchial Lymph Nodes of Guinea Pigs.

No. of animals examined.	No. of tubes inoculated.	No. of tubes showing growth.	Kinds of organisms.	
11	63	42 (66 $\frac{2}{3}$ per cent).	Streptothrix,	28.
			<i>B. subtilis</i> ,	11.
			Cocci,	1.
			Short rods,	1.
			Pleomorphic rods,	1.

To augment his findings and perhaps throw some light on their significance in the lymph glands a series of cultivations was made. The bronchial lymph glands in the guinea pig are easily visible and small enough that all of them may be used for culture material. A relatively simple technique has been devised with which the liability of contamination has been reduced to a minimum. The results are given in Table VI.

66 $\frac{2}{3}$ per cent of the tubes showed growth. In no instance were all the bronchial lymph nodes from any guinea pig sterile. The organisms encountered are similar to those observed in the lung and probably were forms that had been taken into the alveoli and smaller bronchioles and transported to the draining lymph glands.

The mechanism of phagocytosis of bacteria and other foreign bodies within the lung has been studied by many. The more plausible view of Haythorn (11), Permar (12), and others is that dust particles

and certain bacteria are taken up by endothelial cells from the blood vessels. These cells make their way into the air space, and pass out through the walls entering the lymph spaces and ultimately reach the lymph nodes. This seems to offer a satisfactory explanation for the appearance of certain organisms in the lymph nodes. It has been possible to study certain phases of this mechanism. If spores of streptothrix and finely divided particles of carmine, or the dye and *Bacillus subtilis* are suspended in salt solution and injected intratracheally into guinea pigs, after 1 or 2 hours cells which have taken up both materials can be demonstrated in wet preparations or in stained films from the lungs. Many of these cells in size and general morphology conform to the macrophage. Streptothrix in large numbers may be recovered from the bronchial lymph glands 12 to 18 hours after their injection into the trachea.

Quensel was able to isolate organisms in but a small number of instances from the lymph glands of horses, calves, sheep, and swine. The great differences which have been found in the guinea pig may be explained on the proportionate bulk of the tissue examined. With the larger animals comparatively little material was actually cultured. All the visible bronchial lymphoid tissue was used from the guinea pig. In the large animals the distances to the draining lymph glands are greater and a longer time interval is afforded for the destruction of phagocytosed organisms before the phagocytes reach the draining lymph nodes.

DISCUSSION.

From the preceding experiments it is obvious that the lung is readily invaded by air-borne organisms. It hardly seems possible that organisms the size of streptothrix, the type most frequently found, the molds, or even bacteria of the *subtilis* group are capable of vegetating and multiplying within either the smaller bronchi or alveoli. Even moderate multiplication would doubtless lead to serious mechanical disturbance. It seems more reasonable to assume that the spores of these various types which abound in dry vegetable matter are taken into the respiratory tract with each inspiration. During deeper breathing a number must reach the smaller air passages and alveoli. The statement that they reach the small bronchioles and alveoli

appears warranted when it is considered that only borders furthest removed from the principal bronchi were used for cultivation. Tufts of streptothrix were observed to grow through the pleura as well as the cut surfaces, evidently in these instances they were closer to the pleura and grew from alveoli.

The organisms are non-pathogenic when injected subcutaneously. The spores then are comparatively inert and are taken care of by the same mechanism that functions in the case of coal dust and other inert matter. This seems to explain their presence in the bronchial lymph nodes. It may be argued that the cells responsible for phagocytosis do not in all instances destroy the injected spores promptly. Some certainly reach the lymph glands in a viable condition and are doubtless ultimately destroyed there. Rous and Jones (13) were able to show experimentally that living phagocytes were enabled to protect injected organisms from the action of destructive substances in the surrounding fluid. Briscoe (14) in his experiments on phagocytosis in the lungs following intratracheal injection of bacteria and foreign blood corpuscles showed that after 1 or 2 hours the non-pathogenic organisms and red cells had been taken up by mononuclear cells. The polymorphonuclear leucocytes did not appear to any great extent until later. Briscoe's interpretation of the origin of these mononuclear cells differs from that of Haythorn and Pernar.

It has been possible with calves to augment the reported findings. The most frequent organisms encountered in the normal nasal passages of calves have been streptothrix and molds. It has been possible to cultivate similar organisms from the trachea. The experiments in which mixtures of carmine and spores and carmine and vegetative forms were injected into the trachea and in many instances taken up by large cells with single nuclei are additional evidence for the hypothesis. The recovery of streptothrix from the bronchial lymph glands in large numbers 12 hours after the injection into the trachea is also significant.

Cocci of various kinds have been cultivated in a relatively small proportion of animals. The assumption has been to regard them as originating in the upper respiratory tract.

There is no reason to assume a different route of entrance in the case of usual types of animal pathogens. The cilia and other factors

are probably no more capable of keeping out the small vegetative pathogens than the spore forms of the non-pathogenic varieties.

The observations have some practical bearing for those interested in the study of respiratory disease, especially in species like the rabbit and guinea pig. By withholding spore-bearing substances, such as hay and straw, it is possible to cut down the number of contaminating organisms. This is of considerable value where small, oftentimes indistinct foci of consolidation are met with.

SUMMARY.

It has been possible to show that the lungs of such animals as the calf, rabbit, guinea pig, white rat, and white mouse are readily invaded by organisms. The most frequent types observed in cultures from the border of the lungs have been streptothrix, molds, and bacteria of the *Bacillus subtilis* group. These forms originate in certain dry food-stuffs (hay and straw). By withholding or moistening these materials it has been possible to diminish the number of organisms in the lungs. When these materials have been supplied to mice whose lungs under usual conditions contain only a few organisms, the number of positive cultures increases and is comparable with those of the larger animals. The bronchial lymph glands of all guinea pigs examined developed, in $66\frac{2}{3}$ per cent of the tubes, organisms similar to those obtained from the lungs.

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A NEW METHOD FOR MEASURING THE PRESSURE IN THE PULMONARY ARTERY.

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PLATE 19.

(Received for publication, June 5, 1922.)

The problem of devising a reliable and readily applicable method for measuring the pressure in the lesser circulation has engaged the attention of physiologists since 1852, when Beutner (1) under Ludwig's direction inserted a cannula in a branch of the pulmonary artery in rabbits, cats, and dogs, and recorded the pressure by means of a mercury manometer. In these experiments the chest was open, artificial respiration was employed, and a fairly large section of the pulmonary arterial bed was absent due to the insertion of the cannula in a large branch of the pulmonary artery. It is unnecessary for us to list the work of other investigators who have used a similar or closely related technique. Tigerstedt (2) and, more recently, Wiggers (3) have provided critical summaries of all such experiments.

In order to meet the requirements of experiments projected in this laboratory a simple and reliable method for taking pulmonary arterial pressure was required, and this method must be applicable under the following conditions. (a) The experimental circumstances must be those used by us in 1921 (4, 5). In this method the heart and great vessels are exposed—the pulmonary artery being naturally included down to its exit from the pericardial sac—but the lungs are closed in the pleural cavity by sewing the edges of the pericardium to the opening in the anterior wall of the chest. Animals so prepared are able to breathe normally. (b) Cannulation of the pulmonary artery must be attained without circulatory embarrassment and without diminishing the size of the capillary bed of the lungs. (c) The cannula used must be simple of insertion and must be so constructed as

to permit usage over long periods of time without danger from blood clotting.

In their essential features all of these requirements have been considered by former investigators. Knoll (6), in 1888, devised a clamp which apparently isolated a fold of the pulmonary artery through which he inserted a specially devised cannula. Tigerstedt (2) found it difficult to visualize Knoll's apparatus from the published description, and with this difficulty we are in entire agreement. Knoll apparently used a trocar type of cannula combined with a special retaining clamp, but on his own admission, many animals were lost while adjusting this apparatus, and it is also apparent that the pulmonary artery suffered a certain degree of narrowing through its use. Employing rabbits, Knoll did, however, succeed in inserting his cannula and measuring pulmonary blood pressure under normal breathing, the chest having been closed following the placing of the cannula. Henriques (7), in 1893, described his procedure for making pressure measurements in the pulmonary artery in the following terms.

For these investigations I have used dogs, cats, and rabbits, always under artificial respiration, either after curarization or medullary section. The left pleural cavity was opened and a sufficient amount of the thoracic wall removed to lay bare the heart. The pericardium was then opened and the main stem of the pulmonary artery pierced with a stilette of small caliber. That absolutely no bleeding occurs is due to the elasticity of the vessel wall and to the low pressure in the pulmonary artery.

Mellin (8), in 1904, employed a slight modification of the cannula devised by Ludwig. His instrument was a simple tube with a small plate at the end which entered the artery. A second plate of similar type, sliding on the shaft of the cannula and arranged so that it could be clamped in place, closed the opening in the pulmonary artery. In order to insert this cannula the blood flow through the artery was shut off for a brief time, but after practise the period of obstruction was so reduced as to be of little moment. The cannula was defective for continuous use on account of blood clotting, but Mellin obtained many useful measurements of pressure with it.

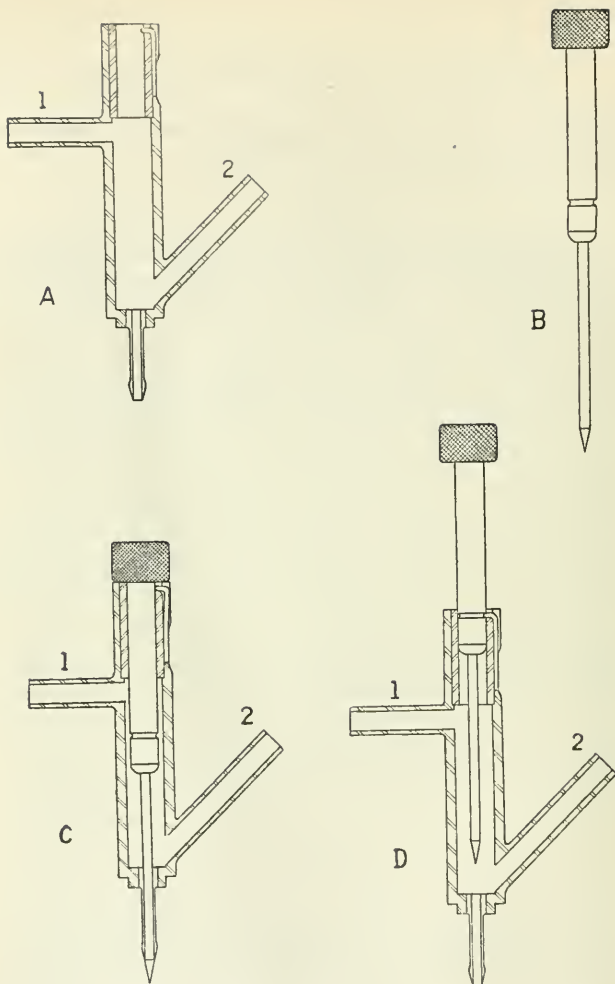
Schafer (9) has recently described a glass cannula for obtaining pulmonary arterial pressure, which is inserted through the wall of the right ventricle. The tip is passed through the pulmonic valve, the leaflets of which close around it. The cannula is provided with a stilette and with side tubes for attachment of pressure-recording and washout apparatus. We have found Schafer's cannula easy to make, easy to insert, and easy to use over long periods of time. We have, however, been unable to employ this cannula without lowering systemic blood pressure to a considerable degree. Even when the greatest care is used the filling of the left ventricle is apparently lessened and the general circulatory condition of the animal suffers. In Schafer's (9-12) publications in which he has employed his cannula, no figures are given covering the systemic blood pressure before and after cannulation of the pulmonary artery. The investigations he has reported

required no such control. If, however, one projects experiments in which the entire circulation must be kept in as normal condition as possible, Schafer's cannula, at least in our hands, does not solve the problem.

Method.

The cannula shown in Text-fig. 1 combines the ideas of Henriques (7) and Schafer (9). It has two parts, a barrel (*A*), and a stilette (*B*). Side tubes enter the barrel, 1 leading to the pressure-recording device and 2 leading to an ordinary washout system. In *C* the cannula is shown ready for insertion. The stilette is well covered with stop-cock grease and makes joints capable of holding water under a pressure of 100 mm. of mercury both at the tip and at the knurled head. To insert the cannula¹ one simply grasps the pericardial tissue on both sides of the pulmonary artery with hemostats and pushes the cannula through the wall of the artery just as a needle is inserted in a vein. As a usual thing no bleeding occurs, and if oozing does take place it soon stops. The cannula is held in place by means of a special clamp upon a ring-stand, and tubes 1 and 2 are connected with the manometer and washout systems. The cannula is then filled with the anticlot solution employed, and all is in readiness to make a record of pulmonary arterial pressure. In order to connect with the blood stream the operator opens the tube leading to the washout system through 2 and withdraws the stilette until the light spring upon the side of the barrel engages the groove in the stilette. This position is shown in *D*, Text-fig. 1. The tube to the washout apparatus is then closed, and on opening tube 1 to the pressure-recording system a tracing is at once obtained. It is obvious that if the stilette is withdrawn so as to throw the barrel of the cannula into communication with the pulmonary arterial stream without opening to the washout apparatus, a vacuum is created which must result in drawing pure blood into the cannula. If, however, the tube to the washout system is open while the stilette is withdrawn, a little additional anticlot is sucked into the system and nothing more occurs. When a suitable length of record has been obtained the tube leading to the washout system is opened and the stilette thrust back into the original position shown in *C*. Under these circumstances

¹ In all the experiments urethane anesthesia was used.



TEXT-FIG. 1. Trocar cannula for pulmonary artery. *A*, the barrel of the cannula; *B*, the stilette; *C*, the stilette in position in the barrel ready for insertion into the artery; *D*, the stilette withdrawn to the notch after insertion into the pulmonary artery. This diagram illustrates the condition of affairs during the actual recording of pressure in the pulmonary artery.

there is no possibility of blood loss or of leakage of anticlot solution into the animal, and the cannula can at once be washed out from the washout system. One may now make another tracing, and the same series of maneuvers may be carried through as many times as may be required. The entire experiment is so simple that it is planned to use it in class work during the coming winter.

Fig. 1 illustrates the beginning of an experiment in a rabbit anesthetized with urethane. Tracing 1 is the pressure in the carotid artery prior to cannulation of the pulmonary artery. Tracing 2 is the carotid pressure after insertion of the pulmonary artery cannula, and tracing 3 is the pulmonary artery pressure taken at the same time. Tracings 1 and 2 are registered by means of a mercury manometer. The mean pressure in tracing 1 is 84 mm. of mercury, and tracing 2 is the same. The mean pulmonary pressure is 18 mm. of mercury. In the experiment in question the rabbit was breathing naturally, since it is easy in this animal to enter the thorax, open the pericardium, and expose the pulmonary artery without opening the pleural cavities.

The entire record shows that one may readily make a measurement, which has been somewhat baffling, without disturbing circulatory relations to a greater degree than is necessitated by exposure of the pulmonary artery. In our experience exposure of the heart and opening the pericardium, whether in the rabbit in which the pleural cavities may be kept shut or in the cat or dog in which they must be opened at least temporarily, result in a lowering of systemic pressure. This is variable in amount and always becomes less when the chest is again closed and the animal begins to breathe naturally.

The validity of pressure measurements made by means of a cannula pointing with the blood current may well be questioned. Theoretically the cannula described should be inserted at right angles to the current in the artery. Such an adjustment can usually be made by cutting away the upper part of the left chest wall where it joins the sternum. We have, however, found that if in the same animal one makes three records—(a) with the cannula pointing toward the heart and directly against the blood current, (b) with the cannula pointing at right angles to the current, and (c) with the cannula pointing in the direction of the blood stream—the results are practically identical and it is therefore unnecessary to observe any particular precautions to avoid error.

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EXPLANATION OF PLATE 19.

FIG. 1. Tracings 1 and 2, pressure in the carotid artery of a rabbit registered by means of a mercury manometer before and immediately after insertion of the pulmonary artery cannula. Tracing 3, pressure in the pulmonary artery registered by means of a membrane manometer.



FIG. 1.

(Swift, Haggart, and Drinker: Pulmonary artery.)

EXPERIMENTAL RICKETS IN RATS.

VI. THE ANATOMICAL CHANGES WHICH ACCOMPANY HEALING OF EXPERIMENTAL RAT RICKETS, UNDER THE INFLUENCE OF COD LIVER OIL OR ITS ACTIVE DERIVATIVES.*†

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PLATES 20 TO 28.

(Received for publication, May 8, 1922.)

The work on experimental rickets which has been conducted in this laboratory during the past 2 years has included an investigation into the nature of the active curative principle contained in cod liver oil. A large series of variously prepared fractions has been tested for curative activity on rats previously rendered rachitic. As a result of this work, material has accumulated which discloses in an interesting way the sequence of changes which accompanies the restoration of rachitic bone to an approximately normal condition. It is the purpose of this article to describe these changes, leaving the problem as to the chemical nature of the active substance of the cod liver oil, which is still under investigation, for future publication.

Shipley, Park, McCollum, Simmonds, and Parsons (1) first reported that the administration of cod liver oil to rats made rachitic by Diets 2538 and 2677, "deficient in the so called fat-soluble A, or in both that substance and calcium,"¹ leads to a rapid deposition of calcium salts between the cells of the proliferative zone of cartilage. Seven treated animals were studied, the period of treatment varying from 2 to 8 days, and of these, six showed a definite reaction. Only the earlier stages of the reparative process are, therefore, considered in their paper.

* This work was done under a grant from the Commonwealth Fund.

† A preliminary communication of this work has been recently published (Zucker, T. F., Pappenheimer, A. M., and Barnett, M., *Proc. Soc. Exp. Biol. and Med.*, 1921-22, xix, 167).

¹ Shipley, Park, McCollum, Simmonds, and Parsons (1), p. 344.

In a second paper, McCollum, Simmonds, Shipley, and Park (2) have elaborated these observations into a biological test for calcium-depositing substances. The rickets-producing diet recommended is No. 3143, described as containing an abundance of protein of good quality, approximately twice the optimal calcium content, fat-soluble A in quantity sufficient to ensure transient growth and to prevent xerophthalmia, but in its phosphorus content, distinctly below the optimal requirement. On this diet, the cartilage is rendered wholly calcium-free, and the metaphysis only exceptionally contains a few calcified areas. The administration of cod liver oil in sufficient quantity to rats thus rendered rachitic is followed by a recalcification of the cartilage, which may be complete within 5 days. A method for the macroscopic study of this healing is described.

The material for the present study consists of sections of ribs of 52 treated rats, together with numerous untreated rachitic controls and normal animals. We have also examined sections through the knee joints, including the lower epiphysis of the femur and the upper epiphysis of the tibia. In our experience, the changes in the ribs are more easily followed, and there is the advantage that the period of decalcification can be materially shortened.

The routine method of fixation and staining described in previous papers (3, 4) was employed. Diet 84, described by Sherman and Pappenheimer (3), has been found satisfactory for therapeutic tests in that the preparatory cartilage remains permanently calcium-free for a period of at least 2 months, even when growth remains stationary (Figs. 3 to 5). When, however, as exceptionally happens, no growth has occurred during the earlier weeks, the rachitic lesions are apt to be very poorly developed and such animals are unsuitable for curative experiments. Furthermore, as shown by Hess, Unger, and Pappenheimer (5), the addition of even 25 mg. of phosphorus in the form of K_2HPO_4 leads eventually to spontaneous healing. We have also observed that rapid loss of weight, especially when associated with diarrhea, favors the deposition of calcium. This is comparable to the effect of complete starvation, as described by McCollum, Simmonds, Shipley, and Park (6). On Diet 84, the rachitic lesions are always well marked in animals which have shown fair growth. Even when the weight curve is stationary or declining, there is no tendency to spontaneous healing, and the changes to be described may, with a considerable degree of certainty, be ascribed to the curative agent used.

In order to follow these curative changes in detail, it is advisable to describe first the appearances found in ribs of (a) normal animals on complete diet; (b) non-rachitic rats which because of inadequate diet, or for other reasons, have failed to grow normally; and (c) rats on the rickets-producing diet (No. 84).

Normal Ribs from Healthy Rats of Approximately 100 to 150 Gm.

Weight, 2 to 3 Months Old (Figs. 1 and 2).

The costal cartilage is calcified in the central portion to within roughly 0.5 mm. of the epiphyseal line. The resting cartilage presents no peculiarities. The proliferating cartilage zone (*Pr*) begins abruptly, the cells become aligned in orderly columns, separated by abundant, bluish staining, uncalcified matrix. The cells increase somewhat in size as the zone of preparatory calcification is approached. The columns have a conical shape, with the apex directed towards the resting cartilage. The width of this zone, according to Erdheim (7), ranges from 100 to 200 μ .

*Zone of Preparatory Calcification (*Pr*₁).*—This averages four cells in depth, and in normal animals does not exceed six cells. The cartilage cells composing it increase greatly in size. The nuclear staining in our preparations is often very pale, and in many of the cells, no nucleus is visible. The cytoplasm is scanty, ragged, and apparently hydropic. The cells, because of their large size, lie closer together than in the proliferative zone; they are embedded in a completely calcified matrix, staining dark purplish blue with hematoxylin, and black or dark brown with silver nitrate.

Endochondral Ossification and Primary Spongiosa.—The process by which the cartilage cells are destroyed by the invading blood vessels, and by which the primary trabeculae are developed about the scaffolding afforded by the persistence of the calcified matrix between the destroyed cartilage cells, is too familiar to require description. The length and thickness of the trabeculae vary considerably in different normal rats; and we have found the development of the primary spongiosa to be a sensitive index of general growth and nutrition. This is well illustrated by sections from a rat in which there was a cessation of growth during the last week of life, which was reflected in an attenuation of the proximal portion of the tra-

beculae—that portion contiguous to the cartilage and last formed. In older rats, showing a very vigorous growth, the trabeculae are long, stout, and closely apposed, bordered by distinct osteoblasts and separated by narrow vascular channels (Figs. 1 and 2, *Sp₁*). In old animals, after the cessation of epiphyseal growth, or in larger animals, in which arrest of growth is artificially brought about by dietary deficiency, such as lack of fat-soluble A, the trabeculae atrophy or become fused into a transverse plate of bone extending across the cartilage.

Osteoid tissue is virtually absent in the primary spongiosa, the calcium apparently being deposited as fast as the osteoid precursor of the bone is elaborated. Only rarely in silver preparations counterstained with Van Gieson's picrofuchsin, or aqueous safranin, is it possible to make out here and there a narrow rim of osteoid.

Secondary Spongiosa (Figs. 1 and 2, *Sp₂*).—This consists of a sparse number of stout trabeculae, completely ossified, without inclusion of calcified cartilage matrix. They join the cortex obliquely, reinforcing it in the region just distal to the epiphyseal line, and joining with trabeculae of the primary spongiosa. An osteoid border is rarely distinctly seen.

Cortex.—The thickness naturally varies with the size and general development of the rat, and is greater in the middle ribs than in the upper ones. It also increases somewhat as one passes from the region of the chondrocostal junction. The cortex is continued over the cartilage as far as the base of the proliferative zone, at which point there is usually a slight constriction, as a thin layer beneath the fibrous perichondrium. Normally, this continuation is only partly calcified.

As regards the osteoid margin, this is to be found only in places,—most commonly, as Erdheim (7) points out, about the perforating vessels, or on the pectoral surface beneath the periosteum. It is more abundant in young, actively growing animals, and may be entirely invisible in older rats. In general, the differentiation between osteoid and calcified bone is more sharply brought out in silver safranin preparations than in hematoxylin-eosin-stained sections. But even in the silver preparations, a visible osteoid border may be entirely lacking in the normal rat. The average thickness of the normal or

physiological osteoid is given by Erdheim as from 5 to 5.5 μ , but there are, as might be expected, fairly wide variations.

Ribs from Althreptic Rats.

The conditions above described are typical for rats which have been on a full diet containing an abundance of necessary mineral and organic foodstuffs, including vitamins.

In non-rachitic rats on a deficient dietary, or in which failure of growth has occurred because of infection, the finer structure of the rib is considerably modified (Fig. 15).

Zone of Preparatory Calcification.—This is often found reduced to two or three cells. The intervening matrix is densely calcified, as in the normal bone. The chief abnormality is in the endochondral ossification.

Primary Spongiosa.—The trabeculae of the primary spongiosa are short, slender, misshapen, and often defective over considerable areas. The exposed cartilage cells are often surrounded, and even invaded by pale fusiform or polygonal cells, among which syncytial multinucleated cells are occasionally found (Fig. 7). These cells, though morphologically related to osteoblasts seem incapable of performing their osteogenetic function. The osteoblasts which surround such trabeculae as are formed, are flat and inconspicuous.

Cortex.—The cortex is thinner than normal, especially in the region of the chondrocostal junction, and the endosteal and periosteal osteoblasts are either missing entirely, or, like those which border the trabeculae, are endothelium-like.

Secondary Spongiosa.—A secondary spongiosa is often wanting. Evidences of increased resorption in these bones are not striking. Occasionally, multinucleated osteoclasts are seen beneath the periosteum, lying in lacunae. The marrow cavity is wide in proportion to the size of the rib. There may be marked congestion, or even hemorrhage, and in infected rats (rat typhoid), areas of focal necrosis are not uncommon.

The appearances described, due in part to deficient osteogenesis, in part to a continuance of the physiological resorption which accompanies bone growth and persists even when growth is arrested, lead to a rarefaction of the bone to which the term "osteoporosis"

is applicable. Our material includes all transitions between extreme degrees of osteoporosis and the normal picture; and as has been stated, it is possible to draw quite accurate inferences as to the general condition of the animal from the histological structure of the ribs, and in particular, from the development of the primary spongiosa.

Ribs from Rachitic Rats.

Although rachitic lesions have been produced on variously modified diets, as reported in previous papers, we need describe here only the appearances observed on Diet 84, which was the diet chosen for the purpose of testing the curative activity of the various cod liver oil fractions.

The gross alterations in the ribs of rachitic rats have been described in detail by Morpurgo (8), Erdheim (7), Pappenheimer (9), Sherman and Pappenheimer (3), Shipley, Park, McCollum, and Simmonds (10), and others, and need not be again recalled. However, an accurate description must be given of the histological changes produced by the diet used in these experiments, in order that the alterations which take place in the course of healing may be made intelligible.

Resting Cartilage.—Erdheim has shown by careful measurements that there is a tendency in rachitic rats for this zone to surpass the normal both in depth and width. The histological structure is, however, in no wise altered.

Proliferating Cartilage.—The boundaries of this zone are difficult to define inasmuch as the failure of calcium deposition in the preparatory cartilage makes it impossible, in the more severely rachitic ribs, to establish a sharp line of demarcation. Where there is a great lateral swelling of the cartilage, the cell columns are often separated by an abundance of matrical substance, in excess of the normal amount. In many of the preparations, the zone of proliferating cartilage, both in respect to its dimensions and the appearance and arrangement of the cells, does not differ notably from that found in normal ribs (Fig. 3, *Pr*).

Zone of Preparatory Calcification.—It is here that one finds the most striking and characteristic changes. On Diet 84, as has been previously stated, there is complete failure of calcium deposition, so that even in the silver nitrate preparations, the entire cartilage re-

mains permanently free of stainable material (Figs. 3 to 5, Pr_1). The only situation in which there is, at times, still some demonstrable calcium is at the extreme tips of the lateral prolongations of the cartilage. The calcium present there probably represents that which had already been laid down at the time when the rachitic regimen was begun, and which at the time of death had not been completely reabsorbed (Figs. 3 and 4, Ca_1).

In the normal rib, the depth of the zone of preparatory calcification varies within very narrow limits, and rarely exceeds four or five cells. In the rachitic rib, there is an enormous increase in the depth of this zone, so that it is often possible to count 50 or more cells from the base of the zone to the tip of the prolongations where the cartilage cells become intermingled with the osteoid tissue of the rachitic metaphysis. The degree of enlargement varies considerably, depending upon the length of time that the rat has been upon the rachitic diet, and also to a certain degree, upon the general growth and nutrition. It has repeatedly been pointed out that rachitic lesions are more severe in well nourished infants than in stunted, marantic ones; and the same differences, in general, hold for the experimental rickets of rats. But even in rats which have shown only transitory gain in weight, the enlargement in the zone of preparatory calcification is a constant and dependable feature. In the study of several hundreds of rats maintained on this diet, we have observed only three rats in which this increase in depth of the cartilage did not greatly exceed the normal limits. These exceptions were in stunted rats suffering from diarrhea during the experimental period, and showing from the outset a stationary or declining weight curve.

The columnar arrangement of the cartilage cells is usually preserved in the basal portion of this zone, a point of importance, as will be shown in the reconstitution of the epiphyseal line in the later stages of healing. Towards the diaphysis, the alignment of the cells in columns is entirely lost, and one encounters great variations in size, shape, and staining. The uncalcified matrix also shows variations in its staining reactions and comes to resemble in places the osteoid tissue which forms so large a component of the rachitic metaphysis. This occurs particularly in the vicinity of the perforating vessels. We shall not discuss here the difficult question as to the direct metaplasia

of cartilage into osteoid tissue, since this has no direct bearing upon the process of healing; and it matters very little how one interprets the transitions which undoubtedly occur between atypical cartilage and osteoid tissue.

It is generally held that the calcification of the cartilage is a necessary condition for the vascular destruction of the cartilage cells. The failure of calcium deposition in rickets may, therefore, be held responsible for the persistence of the cells, and the great increase in the depth of this zone. The formation of new cartilage continues, though probably at a diminished rate,² but because the newly formed cells are no longer opened in orderly fashion by the blood vessels of the marrow, and because of the failure of matrical calcification, there is produced an enormously deepened zone of cartilage cells.

Primary Spongiosa.—After 4 weeks on the rachitic diet, there is formed in the region of the original primary spongiosa an excessive amount of calcium-free osteoid tissue. There is no longer a system of orderly parallel trabeculae ranged in the long axis about the calcified continuations of the cartilage matrix, but broad convoluted masses, many times thicker than normal trabeculae, and often so fused and distorted that their relation to the original trabeculae is wholly obscured (Fig. 3, Sp_1). Some of these osteoid masses still contain a core composed of osseous tissue, inclosing in turn the remains of calcified matrix. Many of them appear to contain no calcified tissue whatever, although it is evident that the section may pass wide of the central bony core. Often there are found columns or irregular groups of cartilage cells embedded in the osteoid tissue.

The newly formed osteoid is in most places homogeneous in texture and stains uniformly and deeply with eosin, or with acid fuchsin in Van Gieson's stain, or with the aqueous safranin in the silver preparations. The demarcation between the calcified bone and the osteoid is invariably a very sharp one. In the process of recalcification, as will be pointed out, the demarcation is obscured.

Perichondral Osteoid.—In the normal rat rib, as described, the cortex extends over the cartilage as far as the base of the proliferative zone, as a thin lenticular strip interposed between the fibrous peri-

² Erdheim (7), p. 102.

chondrium and the cartilage. It is at least partially calcified, the calcium being laid down contiguous to the cartilage. In the rachitic rib, the perichondral osteoid is not only free from calcium, but forms a very thick crescentic mass which contributes largely to the fusiform or bulbous swelling at the junction (Fig. 5, *Pc-ost*). When the rib is angulated, this perichondral osteoid and its continuation as cortical osteoid increase still more and fill in the V-shaped gap on the pectoral aspect.

Cortex.—The calcified cortex (Fig. 5, *Ca*) is characteristically bordered on both its endosteal and periosteal surfaces by a mantle of osteoid which in thickness far surpasses that visible in the normal rat of corresponding age (Fig. 5 *Co-ost*). While this excessive production of osteoid is most extreme in the vicinity of the epiphysis, it extends along the whole length of the shaft, and may be so abundant as materially to narrow the bore of the marrow cavity. The calcified portion is always reduced in thickness, and, rarely, the calcified bone may be entirely interrupted over short stretches, and replaced by osteoid.

Infractures, rather rare except in the more extreme cases of human rickets, are almost regularly produced upon this diet. There is formed about the site of fracture, a globular or fusiform mass of callus, composed partly of cartilage, partly of osteoid tissue. The marrow in the region of the fracture undergoes fibrous transformation. The callus, on this rickets-producing diet, remains free from calcium; the deposition of calcium in the cartilaginous and osteoid callus takes place only under the influence of some curative agent (Figs. 11 and 17).

Marrow and Blood Vessels.—Congestion of the blood vessels in the region of the epiphysis, upon which much stress has been laid by those who believe in the toxic or infectious origin of the disease, is by no means a feature of the experimentally produced rat disease. The overproduction of osteoid in the metaphyseal region is usually so great as to reduce the spaces between the trabeculae to narrow clefts, so that a marked dilatation of the blood channels is mechanically impossible. Even in the earliest stages, hyperemia is not a striking feature, and the vascularity of the epiphyseal region does not exceed that in normal bones.

There is very little cellular marrow interposed between the blood

sinuses and the osteoid trabeculae, in the region of the metaphysis; frequently the endothelium rests directly upon the osteoid tissue. Usually it is possible to distinguish from the endothelium the osteoblasts which border the osteoid trabeculae. They are ranged in orderly or continuous rows, as in normal osteogenesis; but they may be heaped up or lie partly embedded in the osteoid tissue. Fibrous transformation of the marrow is not a conspicuous feature, except in the neighborhood of fractures.

The blood vessels fail to penetrate the cartilage in the even and regular fashion essential to normal ossification, but in the central or axial portion of the rib, they may extend a considerable distance towards the base of the zone of preparatory calcification (Fig. 4, Bv). As has been already noted, the matrix of the cartilage in the vicinity of these penetrating blood vessels tends to stain with eosin, and to resemble osteoid tissue.

We have described the salient features of the rachitic rib somewhat in detail, without, however, entering into a discussion of disputable points and theoretical interpretations. The reader is referred to the exhaustive treatise of Erdheim for a more minute analysis of the rachitic rat lesions.

Healing under the Influence of Cod Liver Oil and Its Active Fractions.

The material at our disposal for the study of the healing process may be grouped as follows:

Beginning healing.....	17 rats.
Advanced healing.....	27 rats.
Healing practically completed.....	8 rats.

The stage of healing depends, of course, not merely upon the length of time that the rat was under treatment, but also upon the activity of the preparation used, and to a certain extent upon the dosage. There are also individual variations, so that identically treated rats do not always show the same degree of healing.

We have selected from our records the following five protocols, as typical of the various stages observed.

Rat 197.—At the age of 4 weeks, the rat was placed upon Diet 84. A radiogram taken 25 days later showed a broad defect in the swollen head of the tibia. After

35 days on the rachitic diet, treatment was begun with daily doses of 5 drops of cod liver oil, Fraction 7, a preparation having only slight curative activity. Radiogram taken after 14 days showed a faint and narrow transverse band extending across the defect in the head of the tibia. The animal was killed and autopsied. Initial weight 45 gm. Maximal weight 54 gm. Terminal weight 48 gm.

Rib.—There is advanced rickets, with great increase in the zone of preparatory calcification, and marked thickening of the perichondral, subchondral, and cortical osteoid. The following evidences of early healing are found.

The zone of preparatory calcification is not entirely free from calcium, but shows, in silver staining, a deposition of lime salts in the lateral portions, extending to within five or six cells of the base, and distally to the terminal cells. There are also irregular deposits in the mid-zonal portion, not extending completely across the cartilage. There is an early calcification of the osteoid of the perichondrium, in the form of fine bluish granules laid down contiguous to the cartilage. Throughout the mass of osteoid tissue which forms the rachitic metaphysis and which envelops the old calcified bone of the cortex with a thick irregular mantle, one may find areas of fresh calcification, distinguishable from the previously deposited calcium by its fine powdery character, and somewhat fainter staining with hematoxylin.

Diagnosis.—Rickets. Very early healing.

The following protocol also illustrates an early stage of healing.

Rat 664.—Received Diet 84 for 28 days. Radiogram at this time showed pronounced rickets. Treated for 7 days with cod liver oil, Fraction 22. After 5 days, the radiogram showed a distinct narrow transverse band indicative of early healing.

Rib (Fig. 6).—The proliferative cartilage shows no changes of consequence. The zone of preparatory calcification averages about 35 cells in depth. The columnar alignment is well maintained in the basal portion. Here the matrix is entirely devoid of calcium and takes a bluish stain. The distal half of the cartilage is traversed by a band of calcification (Fig. 6, *Ca*) taking a purplish stain which contrasts sharply with the uncalcified bluish matrix. The terminal portion of the cartilage, however, is almost free of calcium. In the region of the cartilage where calcification has occurred, there are numerous large blood vessels communicating with the blood sinuses of the marrow (Fig. 6, *Bv₁*). Many of the calcified cartilage cells are being invaded and destroyed, the calcified capsule becoming eroded on one side of the cells, and the space occupied by the degenerating remains of the cartilage cells is filled with red blood corpuscles. The invading vessels apparently lose their endothelial lining, or at any rate appear to discharge their red corpuscles into the empty capsular space. In a few of the eroded cartilage cells, one finds a group of three or four small angular cells, staining deeply with hematoxylin and resembling osteoblasts in their morphology.

Very interesting changes are seen in some of the unopened cartilage cells which lie in the calcified zone. The cytoplasm is filled with hyaline droplets of various sizes, staining deeply with eosin, and resembling those seen in degenerating renal epithelium (Fig. 18). The nucleus of these cartilage cells is sometimes normal, more commonly shrunken and irregular in outline.

The spongiosa is represented by irregular trabeculae composed of calcified bone, osteoid tissue, and atypical or metaplastic cartilage. Closer study shows certain differences from the picture found in untreated rats. The osteoid tissue is less homogeneous than usual, more fibrillar, and there are clefts and fissures in which lie small angular osteoblasts or bone corpuscles. The surface of the trabeculae is not smooth, but irregularly eroded. The osteoblasts do not cover the trabeculae in continuous rows, but are heaped up in places on the surface, or partly embedded in the osteoid.

The greatly thickened perichondral osteoid shows recent calcium deposits in several places (Fig. 6, *Ca*). Adjacent to the cartilage, there is a fringe of calcium, the granules being deposited in rows following along the course of the fibrils. Further out nearer the fibrous perichondrium, there are also irregular areas of calcification, easily distinguishable from the previously calcified bone by their granular character.

A very striking feature of the section is the distention of the large blood sinuses in the subchondral region (Fig. 6, *Bv₂*). The trabeculae of the spongiosa are separated by these dilated blood sinuses, very few of the blood-forming elements being interposed between the endothelium of the sinuses and the trabeculae. Further along the marrow cavity, this dilatation of the blood channels becomes progressively less marked, and the marrow resumes its normal vascularity.

The cortical osteoid is very greatly increased in amount. Here and there, especially on the periosteal surface, there are areas of granular calcification.

Summarizing the evidences of healing, we find (1) deposition of calcium in the distal half of the widened zone of preparatory calcification; (2) invasion of the calcified portion from the lateral and subchondral sides by large blood vessels, with destruction of the cartilage cells; (3) changes in the osteoid tissue which may be taken to indicate beginning lysis or halisteresis; (4) great dilatation and congestion of the blood sinuses in the metaphyseal region; and (5) deposition of granular calcium in the perichondral and pericortical osteoid.

A later phase of healing is presented by the ribs of Rat 660.

Rat 660.—After 28 days on Diet 84, marked rickets had developed, as shown by the radiogram. Cod liver oil, Fraction 23, was then given daily for a period of 36 days. During the last 2 weeks of life, the weight declined from 73 to 58 gm., which may have favored the healing process. A radiogram, taken on the 9th

day after beginning treatment, that is before the rat had begun to lose weight, showed a delicate transverse band crossing the tibial defect.

Rib (Fig. 10).—Macroscopically, there is still marked fusiform swelling at the chondrocostal junction. The following conditions are found in sections.

The zone of proliferating cartilage is somewhat reduced in depth; the columns are short and widely separated (Fig. 10, *Pr*). The zone of preparatory calcification (Fig. 10, *Pr*₁) consists of two fairly distinct portions. The basal part is composed of orderly columns for a distance of six or eight cells, between which the matrix is completely calcified. The calcium ends in an even line at the base of the zone of proliferative cartilage, and no blood vessels penetrate this portion. From this basal part, there extend shaftwards into the original rachitic metaphysis large irregular processes of cartilage, in which a columnar arrangement of the cells is for the most part lost; and in the distal portion, individual cartilage cells become separated from one another by a pink-staining matrix. There is very extensive calcium deposition throughout these prolongations; it is only where the matrix shows this atypical staining and merges into osteoid tissue, that no lime salts have been laid down. These prolongations of cartilage are being invaded from all sides by wide congested blood vessels (Fig. 10, *Bv*), so that the entire metaphysis has a coarse spongy structure. Wherever the cartilage cells are in contact with the blood vessels, they are being eroded in precisely the same fashion as during the process of normal endochondral ossification, save that the cartilage cells are attacked from all sides. Comparatively little bone is formed about the calcified remains of the matrix, but here and there, especially about the basal portion of the cartilage, one finds groups of unopened cartilage cells, surrounded by an irregular shell of osseous tissue, bordered by distinct osteoblasts. In general, no osteoblasts or marrow elements are interposed between the blood channels and the calcified cartilage.

Towards the shaft, the section passes tangentially to one side of the marrow cavity, so that one comes rather suddenly upon a solid mass of osteoid tissue. Further along, the marrow cavity is again exposed.

The osteoid tissue, wherever seen, is in the process of becoming recalcified. The old calcified bone takes a bluish stain, and contains bone corpuscles heavily impregnated with hematoxylin, but this previously calcified bone shades off gradually into a granular purplish fringe of recently deposited calcium, so that the demarcation between calcified bone and uncalcified osteoid is greatly obscured. This is brought out also in the silver preparations, where the old bone takes a compact black stain, but the newly deposited calcium salts at the margin appears as discrete granules. An oval clear space is left about the bone corpuscles in the calcifying osteoid, whereas minute angular clefts of much smaller size mark the site of the adult bone corpuscles in the originally calcified bone.

The salient features in this rib are therefore (1) a complete recalcification of the enormously widened preparatory cartilage; (2) active

erosion of this calcified cartilage by large blood channels, leaving, however, a basal columnar layer, approximately six cells in depth, which, as shown by the study of subsequent stages, is destined to form a new zone of preparatory cartilage; and (3) extensive recalcification of the osteoid tissue, perichondral, subchondral, and cortical, with its conversion into adult bone. Evidences of active bone formation or of increased bone destruction are not striking.

The final stage in the healing process is exemplified by Rat 846.

Rat 846.—This rat was placed on Diet 84 for 32 days. Radiogram taken on the 31st day showed the usual marked rickets. Daily treatment with 5 drops of cod liver oil, Fraction 31-D, was then given for a period of 19 days. Radiogram on the 11th day of treatment showed a well marked transverse shadow. The animal gained only 4 gm. during the entire experimental period and was poorly nourished at autopsy.

Rib (Fig. 14).—There is still slight fusiform swelling visible grossly and in the sections. The zone of proliferative cartilage (Fig. 14, *Pr*) is somewhat narrower than in normally growing rats. The zone of preparatory calcification has been completely reformed (Fig. 14, *Pr*). It is composed of orderly columns of three or four swollen cells, separated by a densely calcified matrix which merges directly into the primary spongiosa (Fig. 14, *Sp*₁). The latter consists of a series of short broad trabeculae, some of which extend over several rows of cartilage cells, and are partially fused at their distal ends. In the recesses between the trabeculae, loops of capillaries extend upwards in contact with the cartilage cells, some of which are being eroded, but in a less orderly way than in normal actively growing bone. The capillaries are accompanied by a few pale osteogenetic cells, in which mitotic figures are occasionally found.

The fusiform swelling distal to the cartilage is enclosed by a thickened cortex (Fig. 14, *Co*), derived from the ossification of the rachitic osteoid. There is an abundant secondary spongiosa (Fig. 14, *Sp*₂), which consists of many irregularly disposed trabeculae. A few of these still contain one or several cartilage cells. For the most part, however, these trabeculae are completely changed into bone. The osteoid tissue has thus been in part resorbed, in part completely calcified and converted into bone.

The blood sinuses in this region are enormously widened and crowded with red cells. An endothelial lining is not always distinct, but this may be a fault in preparation. Between the distended blood channels, there is a fair amount of normal cellular marrow, but in many places, the vessels are in direct contact with the marrow.

The cortex in hematoxylin-eosin preparations takes a uniform blue stain; but in silver-safranin slides, there is still found in places, especially about the perforating vessels, and along the endosteal surface, a border of uncalcified osteoid distinctly wider than the normal.

The rachitic lesions, revealed by the radiogram before treatment was begun, have been followed by an almost complete restitution to the normal. There are still some detectable traces of the previous rickets; namely, the fusiform swelling at the site of the original rachitic metaphysis, the presence of a few included cartilage cells deep in the spongiosa, the disorderly arrangement of the bony trabeculæ, and the presence of osteoid in slightly excessive amount along the cortex. The epiphyseal line, however, has been completely reconstituted, and healing is virtually completed.

The following protocol is cited to show that with the healing of the rachitic lesions an extreme osteoporosis may develop.

Rat 653.—Placed on Diet 84 for 28 days. Radiogram taken on the 27th day showed pronounced rickets. Treatment with cod liver oil, Fractions 21 and 24 was administered over a period of 50 days. Radiograms taken on 5th, 10th, 36th, and 49th days after beginning treatment show the progress of the healing (Fig. 16). The rat increased in weight from 42 gm. at the beginning of the experiment to 56 gm., but lost 13 gm. during the remaining period of life.

Rib (Fig. 15).—The picture is that of an extreme osteoporosis. The zone of preparatory calcification is reduced to two cells in depth, the matrix between the cells is densely calcified. There is practically no primary or secondary spongiosa (Fig. 15, Sp_1 , Sp_2), only two or three thin, obliquely placed trabeculæ remaining. The cortex (Fig. 15, Co) is thin, completely calcified, save for an even narrow strip of endosteal osteoid bordered by distinct osteoblasts. The marrow cavity is wide and the sinuses are congested. The cellular marrow extends to the cartilage.

In this rat, the previous rachitic lesions, evident in the radiogram, have completely healed and given place to an extreme osteoporosis.

DISCUSSION.

From these illustrative protocols, and from a study of numerous other examples, it is possible to trace quite logically the sequence of events in the healing process.

The first obvious effect of the administration of cod liver oil or one of its active fractions in the ribs of a rat rendered rachitic by Diet 84, is the deposit of calcium salts in the zone of preparatory calcification. The exact site of this deposition varies somewhat, but it begins regularly in the lateral aspects of the cartilage, and, somewhat later, extends across the cartilage as a broad band. Gradually, the area

extends basally as far as the rows of proliferating cells, and distally it involves all the irregular prolongations throughout the rachitic metaphysis (Fig. 9).

The time relations vary greatly—with the dosage, with the activity of the preparation used, and probably also with the severity of the rachitic lesions at the time when treatment was begun. We have observed beginning calcification within 24 hours after the administration of a single dose of pure cod liver oil, and after 5 to 7 days, calcium is often present throughout the greater portion of the cartilage. The complicated rearrangements necessary to bring about a complete return to the normal require, however, a considerably longer period.

Accompanying the deposition of calcium in the matrix of the cartilage, there is a laying down of the salts also in the osteoid tissue. In the perichondral osteoid, which is always very considerably thickened, one finds a granular deposit beginning in that portion of the osteoid contiguous to the cartilage (Fig. 8, Ca_2). The granules of calcium are ranged often in linear rows at right angles to the cartilage, like minute stalactites (Fig. 8, Ca_1). In the trabeculae of the spongiosa and in the osteoid masses which envelop the calcified bone of the cortex, new calcium is laid down first in granular form, or, as seen in hematoxylin-eosin preparations, as a faint purple cloud fringing the originally calcified bone. As this osteoid tissue becomes transformed into fully calcified bone, the osteoblasts embedded in its substance change their character, becoming more pycnotic and angular and acquiring the stainable processes distinctive of adult bone corpuscles.

This active deposit of calcium in the cartilage and osteoid is very easy to demonstrate and to understand, at least from the morphological view-point. But the return to the normal structure involves also a resorption of the excessive cartilage and osteoid tissue which make up the swollen rachitic metaphysis; and there are details in this process which are less easily analyzed.

The excess of cartilage appears to be disposed of in the following way. With the calcification of the capsules and matrix of the cartilage cells, there is an invasion from all sides by blood vessels which bring about a re-solution of the calcium and a digestion of the contained cellular material, exactly in the same way as this takes place

physiologically during endochondral ossification. The only difference is that this destruction of the cartilage cells takes place most irregularly, whereas in normal endochondral growth, the erosion of the cells occurs in uniform and orderly fashion at the line of ossification.

With the disappearance of the cells, there remain for a time portions of undissolved cartilage matrix in the form of curved rods which may form the framework of new bony trabeculae.

It is important to note that this destruction spares the cells at the base of the cartilage, where the columnar alignment is still preserved. Usually rows of four or five cells remain uninvaded by the blood vessels and these basal cells eventually form the new zone of preparatory calcification, when healing is completed (Fig. 13).

The removal of the excess cartilage through the lytic agency of the blood vessels, therefore, involves no new principles. It is apparently conditioned by the calcification of the matrix, and the same factors, presumably, are operative as are concerned in the physiological destruction of cartilage during normal growth. The resorption of the excessive amount of osteoid, which undoubtedly also occurs on a large scale, is less easy to follow. We have observed that at this stage the osteoid tissue still present in the subchondral region stains less intensely, has a looser fibrillar structure, and often appears frayed and eroded at the margins—appearances which suggest that it is undergoing solution. The contained corpuscles, or those on the edge, lie in clefts, as if they were being freed from their imprisonment by the dissolution of the rigid material about them. Occasional multinucleated cells may be found, but osteoclastic resorption certainly plays a very subordinate part in this process. The deeply staining cells which are often found in rows or small groups on the surface of the trabeculae have the character of osteoblasts, and there seems no good reason for assigning to them a rôle in resorption. The exact process by which much of the uncalcified osteoid is removed remains obscure. It is possible that further study with special staining methods may add to our understanding, but the interpretation of the finer changes accompanying resorption has always been a matter of difficulty.

However brought about, this resorption of the osteoid very clearly takes place first in the proximal half of the rachitic metaphysis, that is the portion nearest the cartilage, and only later affects also the distal portion.

Accompanying the removal of the excessive cartilage and osteoid tissue, there takes place also an extreme and striking distention of the blood vessels. This may well be brought about passively by the solution of the solid structures, so that the vascular channels are without support, and converted into veritable lakes of blood (Figs. 12 and 13, *Bv*).

Two stages in the recalcification of the callus about a fracture, under the influence of active preparations of cod liver oil, are illustrated in Figs. 11 and 17. The recalcification of the callus cartilage, and its conversion into spongy bone, are brought about in the way described in discussing the alterations of the cartilage in the zone of preparatory calcification during healing.

The study of the anatomical changes cannot, obviously, explain satisfactorily the mode of action of the cod liver oil. That remains a purely chemical problem. The matter, however, is to a considerable degree simplified by the observation that the determining incident is the initial calcification of the preparatory cartilage and osteoid. The subsequent changes follow inevitably, and are no different in principle from those that take place in normal bone growth. The problem, therefore, resolves itself into the question of how cod liver oil promotes the deposition of the calcium. It would be premature to discuss this in detail, nor does it fall within the scope of this paper to consider the chemical aspects of this healing process.

It is hoped, however, that the above description will supplement the excellent studies of Schmorl (11) and others on the healing of human rickets. While the earlier phases have been accurately described by Schmorl, and correspond in all essentials to those seen in the healing of experimental rat rickets, it is only in the experimentally controlled disease that it is possible to follow the reparative changes through all stages to complete healing.

SUMMARY.

Rats which had developed rickets were treated with cod liver oil or an active fraction of cod liver oil, and the various changes which occur in the healing of the bone lesions were studied. Several phases are described, from the early deposit of calcium salts in the zone of preparatory calcification to the return to normal bone.

These stages correspond closely to those found in the healing of human rickets.

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EXPLANATION OF PLATES.

Rc, resting cartilage; *Pr*, proliferative cartilage zone; *Pr*₁, zone of preparatory calcification; *Pr*₂, prolongations of preparatory cartilage; *Sp*₁, primary spongiosa; *Sp*₂, secondary spongiosa; *Co*, cortex; *Pc*, perichondrium; *Po*, periosteum; *Ost*, osteoid tissue; *M*, marrow; *Bv*, blood vessel; *Ca*, calcium.

PLATE 20.

FIG. 1. Rat 520. At the age of 4 weeks placed on Diet E, consisting of:

Flour.....	80.5 per cent.
Egg albumin.....	10.0 per cent.
Butter fat.....	5.0 per cent.
Complete salt mixture (Z-85).....	4.5 per cent.

After 30 days, the weight had increased from 47 to 90 gm. Normal rib. Hematoxylin-eosin.

FIG. 2. Rat 675. At the age of 4 weeks placed on Diet E-M, containing:

Flour.....	79.7 per cent.
Egg albumin.....	10.0 per cent.
Butter.....	5.0 per cent.
Complete salt mixture.....	5.3 per cent.

The weight increased from 65 to 162 gm. during the period of 48 days. Normal rib. Hematoxylin-eosin.

PLATE 21.

FIG. 3. Rat 811. 27 days on Diet 84. Typical rachitic lesions. Silver nitrate-safranine.

FIG. 4. Rat 816. 47 days on Diet 84. Advanced rickets. No deposition of calcium in preparatory cartilage or osteoid. Silver nitrate-safranine.

PLATE 22.

FIG. 5. Rat 636. 66 days on Diet 84. Initial weight 42 gm.; terminal weight 68 gm.; maximal weight (4 days antemortem) 74 gm. Complete absence of calcium in zone of preparatory cartilage, and in perichondral and cortical osteoid. No tendency to "spontaneous" healing. Silver nitrate-safranine.

FIG. 6. Rat 664. 28 days on Diet 84. Then given daily treatment with cod liver oil, Fraction 22, for 7 days. Beginning calcium deposition in preparatory cartilage and osteoid (*Ca*). Silver nitrate-safranine.

PLATE 23.

FIG. 7. Rat 47. 90 days on diet deficient in fat-soluble A. Moderate osteoporosis. Pale cells about cartilage (*A*); syncytial cells at *S*. Hematoxylin-eosin.

FIG. 8. Rat 110. 27 days on Diet 84. Single dose of 5 drops of cod liver oil, Fraction 4 (active preparation), 24 hours before death. Beginning deposition of calcium (*Ca*₁) in perichondral osteoid (*Pc-ost*), in the form of striate granules perpendicular to the long axis. Calcification of matrix of cartilage (*Ca*₂) in distal portion of zone of preparatory calcification. Hematoxylin-eosin.

PLATE 24.

FIG. 9. Rat 847. 32 days on Diet 84. Then given daily treatment with 5 drops of cod liver oil, Fraction 31-D (diluted but active preparation), for a period of 19 days. Marked healing shown in radiogram. Rib shows extensive recalcification of preparatory cartilage, and of perichondral and cortical osteoid; marked dilatation of blood vessels (*Bv*). Pale cartilage cells may be seen at *A*. Silver nitrate-safranine.

FIG. 10. Rat 660. 28 days on Diet 84. Then given daily treatment with slightly active preparation of cod liver oil, Fraction 23, for a period of 36 days. Rib shows moderately advanced healing. Complete recalcification of zone of preparatory cartilage, with areas of resorption, and great widening of blood sinuses. Calcification of perichondral osteoid. Silver nitrate-safranine.

PLATE 25.

FIG. 11. Rat 189. 36 days on Diet 84. Then given daily treatment with cod liver oil, Fraction 12, for a period of 9 days. Rib shows callus about fracture (*F*), with beginning calcification of cartilage. In untreated rats on Diet 84, the callus remains calcium-free. Silver nitrate-hematoxylin-Van Gieson.

FIG. 12. Rat 647. 30 days on Diet 84. Then given daily treatment with cod liver oil, Fraction 24-A (active preparation), for 11 days. Advanced healing. A large part of the preparatory cartilage, following calcification of the matrix, has been eroded and replaced by wide blood sinuses. Osteoid tissue is still present in the distal portion of the metaphysis. Silver nitrate-safranin.

PLATE 26.

FIG. 13. Rat 646. 30 days on Diet 84. Then given daily treatment with cod liver oil, Fraction 24-A, for a period of 11 days. Advanced healing. Included cartilage cells in secondary spongiosa (*Sp₂*). Hematoxylin-eosin.

FIG. 14. Rat 846. 32 days on Diet 84. Then given treatment with cod liver oil, Fraction 31-D (active preparation), for 19 days. Rib shows virtually complete healing. A new zone of preparatory calcification and spongiosa have been reconstituted, and recalcification is everywhere complete. Traces of previous rachitic deformity are still evident in the fusiform swelling, irregularity of trabeculae, and thickening of cortex at the site of the original rachitic metaphysis. Hematoxylin-eosin.

PLATE 27.

FIG. 15. Rat 653. 28 days on Diet 84. Then given daily treatment with cod liver oil, Fraction 21, for a period of 30 days, followed by Fraction 24 for 20 days. The progressive healing of the rachitic lesions is shown in Fig. 16, *a* to *e*. Initial weight 42 gm.; maximal weight (36th day) 56 gm.; terminal weight 43 gm. Rib shows marked osteoporosis. Zone of preparatory calcification reduced to two cells; matrix calcified; primary spongiosa fused, trabeculae rudimentary; cortex thinned, no visible osteoid border; secondary spongiosa defective. Hematoxylin-eosin.

FIG. 16, *a* to *e*. Rat 653. Radiograms taken before, and on the 5th, 10th, 36th, and 49th days after beginning treatment.

PLATE 28.

FIG. 17. Rat 962. 31 days on Diet 84. 5 drops of cod liver oil daily for 10 days. Rib shows fracture (*F*) with bone formation in callus (*A*). Remains of cartilage at *C*; recalcification of osteoid at *Ca*. Hematoxylin-eosin.

FIG. 18. Rat 664. Hyaline droplets in cartilage cells. Hematoxylin-eosin. Oc. 3, obj. $\frac{1}{2}$. Oil immersion.

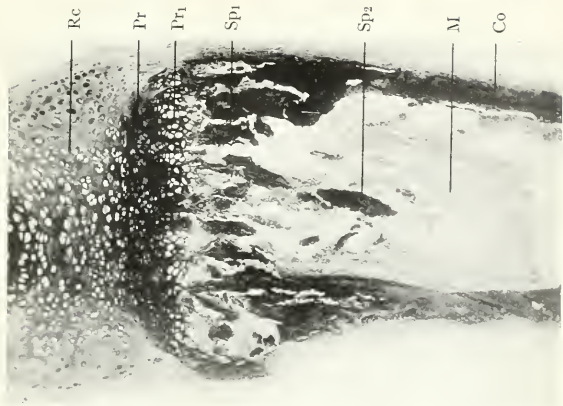


FIG. 2.

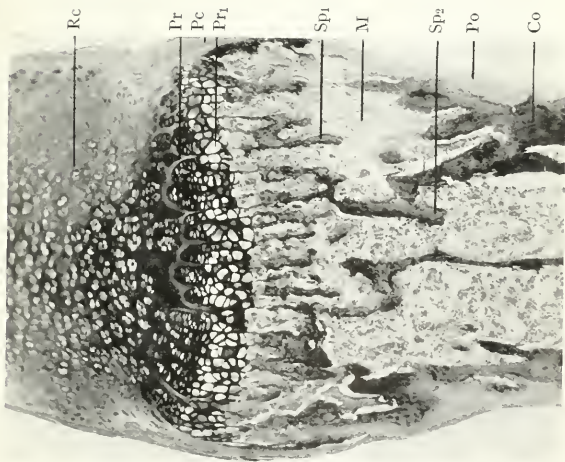


FIG. 1.

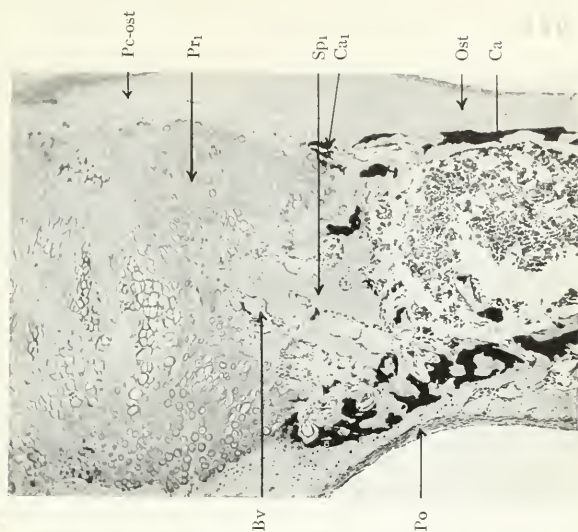


FIG. 4.

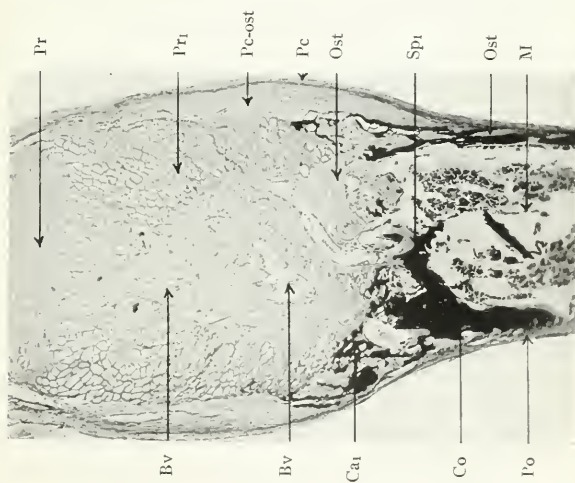


FIG. 3.

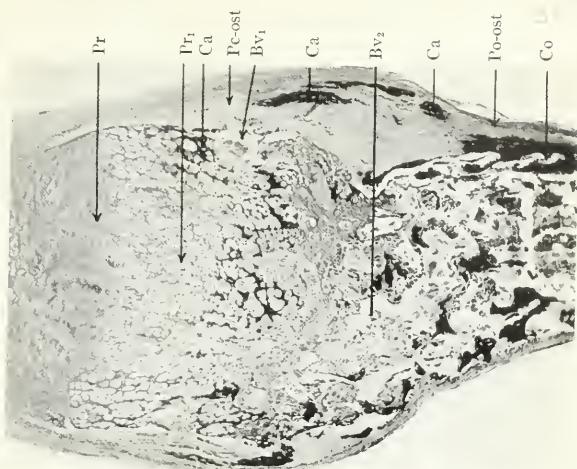


FIG. 6.

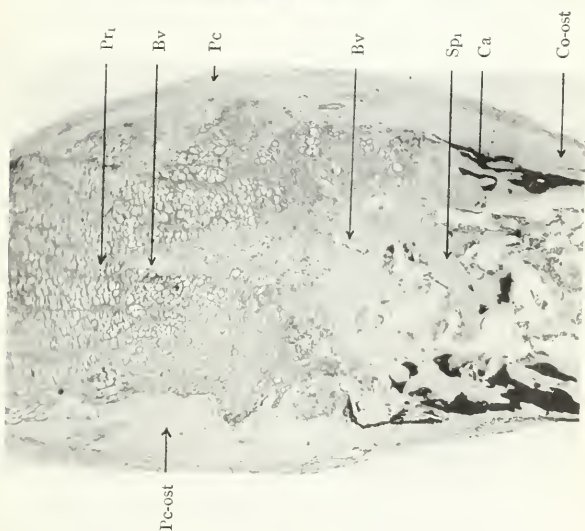


FIG. 5.



FIG. 7.

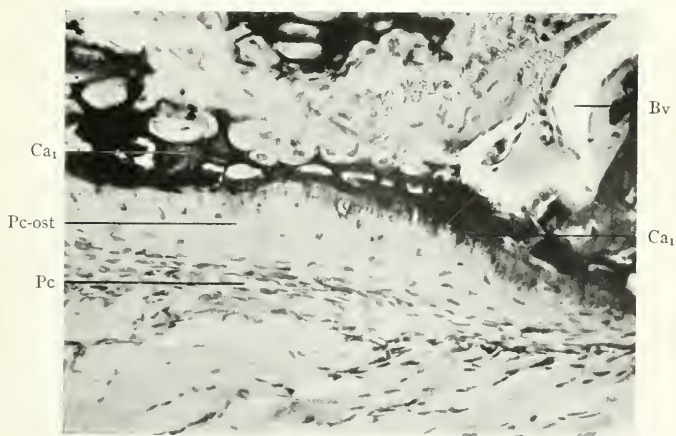


FIG. 8.

(Pappenheimer: Experimental rickets. VI.)

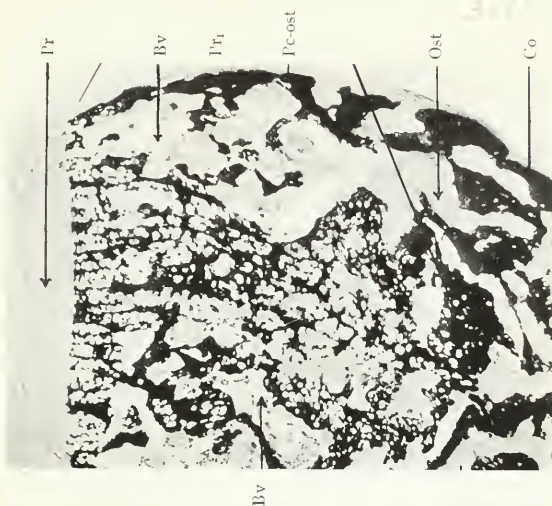


FIG. 10.

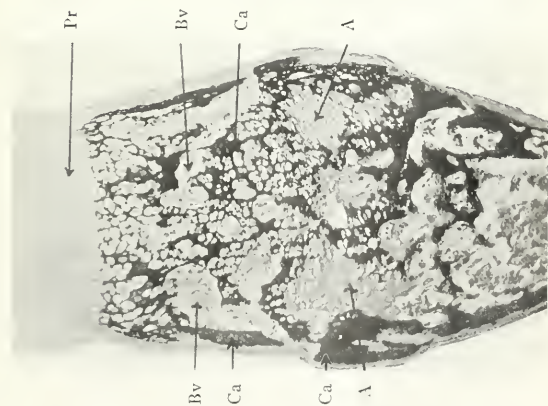


FIG. 9.

(Pappenheimer: Experimental rickets. VI.)

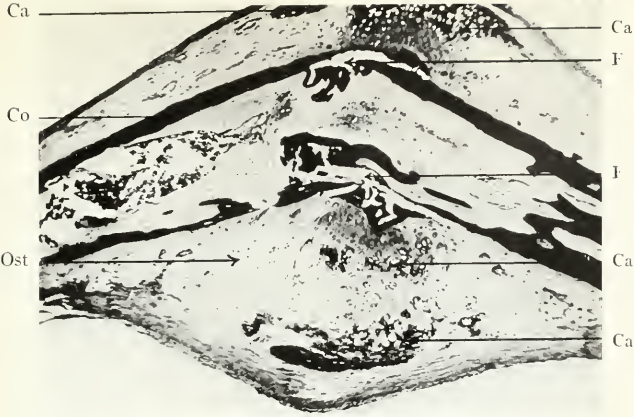


FIG. 11.

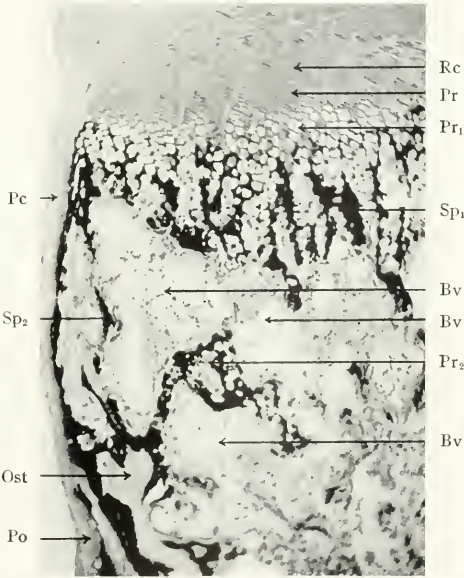


FIG. 12.

(Pappenheimer: Experimental rickets. VI.)

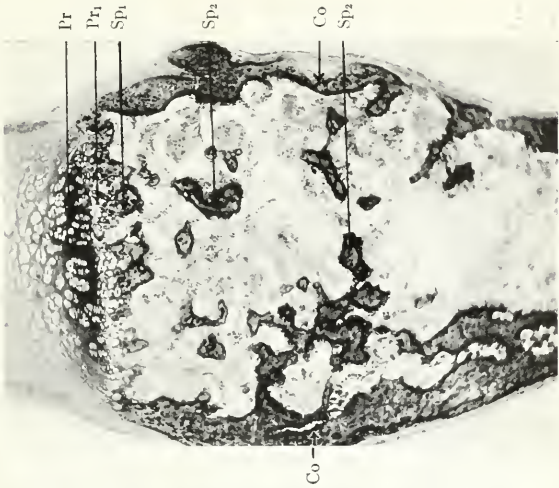


FIG. 14.

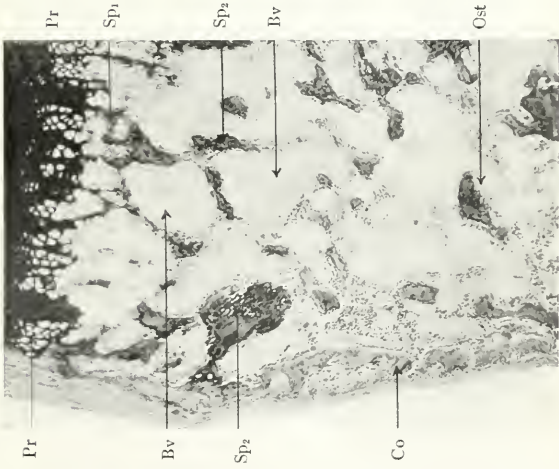


FIG. 13.

(Pappenheimer: Experimental rickets. VI.)

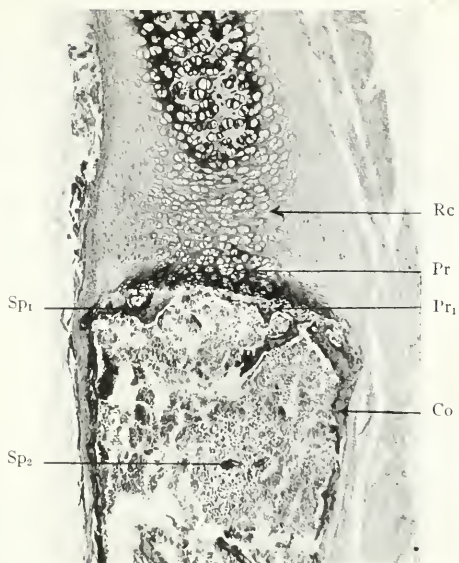


FIG. 15.



a

b

c

d

e

FIG. 16.

(Pappenheimer: Experimental rickets. VI.)

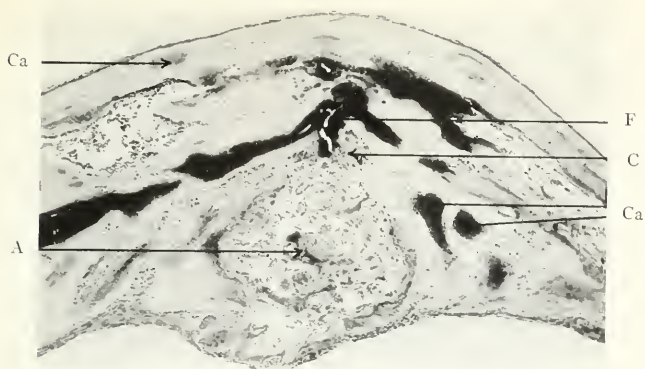


FIG. 17.



FIG. 18.

(Pappenheimer: Experimental rickets. VI.)

ETIOLOGY OF YELLOW FEVER.

XIV. DURATION OF THE PROTECTIVE EFFECT OF ANTI-ICTEROIDES IMMUNE SERUM AFTER SUBCUTANEOUS INOCULATION INTO ANIMALS.

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(Received for publication, May 11, 1922.)

As has already been reported,¹ the anti-*icteroides* immune serum is capable of protecting susceptible animals against infection with *Leptospira icteroides* when administered simultaneously or during the early period of the disease. Lyster, Pareja, Bailey, Vaughn, Vasconcelos, Casassus, Iglesias, Loyo, Le Blanc, Lynn and his associates, Hernandez, Lara, Villamil, Kligler, and Gann² have found that the mortality among human cases of yellow fever treated with the immune serum on or before the 3rd day of disease is much lower than that among untreated cases. Of 71 cases treated within the first 3 days of disease in Guatemala, Salvador, Honduras, Vera Cruz Merida, and northern Peru only 5 died (7 per cent mortality); the results of serum treatment were less favorable in the Tuxpam (Mexico) epidemic, where the mortality among the treated was 25 per cent (9 deaths among 36 cases), but here the mortality among untreated cases was correspondingly higher, being 68.6 per cent (59 deaths

¹ Noguchi, H., Serum treatment of animals infected with *Leptospira icteroides* *J. Exp. Med.*, 1920, xxxi, 159; Chemotherapy versus serotherapy in experimental infection with *Leptospira icteroides*, 1920, xxxii, 381. Noguchi, H., and Kligler, I. J., Immunological studies with a strain of leptospira isolated from a case of yellow fever in Merida, Yucatan, *J. Exp. Med.*, 1920, xxxii, 627; Immunology of the Peruvian strains of *Leptospira icteroides*, 1921, xxxiii, 253.

² Noguchi, H., Prophylaxis and serum therapy of yellow fever, *J. Am. Med. Assn.*, 1921, lxxvii, 181. See also the report of The Rockefeller Foundation for the year 1921.

among 86 cases). Of the total 187 cases treated to date, 107 received the serum on or before the 3rd day, and of these, only 14 died (mortality 13 per cent); on the other hand, there have been 41 deaths (51 per cent mortality) among 80 cases treated on or after the 4th day, and 225 deaths (56.6 per cent mortality) among 397 untreated cases occurring in the same localities during the same epidemic periods.

The serum has apparently undoubted therapeutic value in human cases of yellow fever as well as in cases of experimental infection with *Leptospira icteroides* in animals. Vaccination by means of killed cultures of *Leptospira icteroides* (injected in two subcutaneous injections of 2 cc. each, 4 to 6 days apart) has been shown to confer complete protection within 10 to 15 days of the last inoculation.^{2,3} During the period required for development of active immunity, however, anti-*icteroides* serum might be utilized for the immediate protection of non-immune individuals who find themselves in an epidemic or endemic focus of yellow fever, or it might be substituted for vaccination in the case of persons who intend only to pass through an infected district. It is well known that an immune serum or antitoxin, when introduced into the system of a non-immune individual, will protect against infection for a period which varies from a few to many days according to the number of units of immune bodies initially introduced and the rate of elimination of such bodies from the inoculated individual. The passive immunity thus conferred is necessarily of short duration, yet the employment of anti-*icteroides* serum for the temporary protection of non-immune individuals might be of considerable practical value.

In the experiments to be reported here guinea pigs were used to determine the duration of the protective effect of an injection of anti-*icteroides* immune serum.

EXPERIMENTAL.

Six different doses of immune serum (0.00001, 0.0001, 0.001, 0.01, 0.1, and 1 cc.) were subcutaneously inoculated into six sets (two each) of guinea pigs of about 500 gm. body weight. The tests for the per-

² Noguchi, H., and Pareja, W., Prophylactic inoculation against yellow fever, *J. Am. Med. Assn.*, 1921, lxxvi, 96.

sistence of passive immunity were made 1, 2, 3, 4, 5, 7, 10, and 15 days after the injection of the immune serum. In order to infect all the guinea pigs, including six normal control animals, with the same material at the same time, the injections of the immune serum were begun 15 days before the time selected for the test inoculation of a virulent strain of *Leptospira icteroides*. The infective material used was an emulsion of the liver and kidneys of a guinea pig fatally infected with a strain of *Leptospira icteroides* isolated in Morropon by Noguchi and Kligler,⁴ which killed the control guinea pigs within 9 to 10 days in quantities of 0.001, 0.01, and 0.1 cc. 0.1 cc. of the emulsion, representing about 100 minimum lethal doses, was used in this experiment and was given subcutaneously. The results are recorded in Table I.

Analysis of the table shows that the minimum quantity of anti-*icteroides* serum required for protection, as revealed by the results obtained when the serum was given an hour previous to the inoculation of the infecting material, lay between 0.00001 and 0.0001 cc. Judged from the survival of three guinea pigs which received serum 24, 48, and 72 hours previously the protective titer of the serum must be close to 0.0001 cc. There was no diminution in the protective effect within 48 hours, but after 3, 4, and 5 days 0.0001 cc. no longer gave complete protection, while 0.001 cc. protected in every instance. 7 days after the time of injection of the serum 0.001 cc. failed to protect, but 0.01 cc. was still effective. After the lapse of 10 days 0.1 cc. was required to prevent infection, and after 15 days only those animals which had received 1 cc. of the immune serum withstood infection. The rate of elimination of the immune substance in the body of the guinea pig after the subcutaneous introduction of the anti-*icteroides* serum does not proceed uniformly in the successive days following injection, but follows a characteristic course which may be roughly estimated in the manner shown in Text-fig. 1, the unit being 0.0001 cc., which neutralized at least 100 minimum lethal doses in the present series.

The rate of disappearance of the immune substance is very slow at first, but becomes rapid after about 10 days. This phenomenon is

⁴ Noguchi, H., and Kligler, I. J., Experimental studies on yellow fever in northern Peru, *J. Exp. Med.*, 1921, xxxiii, 239.

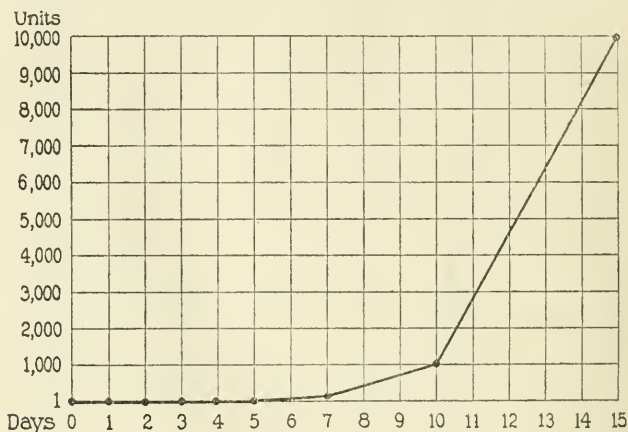
TABLE I.
Results of Inoculation of 100 Minimum Lethal Doses of Leptospira icteroides into Guinea Pigs Previously Injected with Anti-icteroides Immune Serum.

Amount of serum injected.	Intervals between the injection of the immune serum and the inoculation of <i>Leptospira icteroides</i> .								
	1 hr.	24 hrs.	48 hrs.	3 days.	4 days.	5 days.	7 days.	10 days.	15 days.
cc. 0.00001	+	—	—	—	Died in 9 days.	Died in 10 days.	Died in 11 days.	Died in 11 days.	Died in 10 days.
	(Killed for virus.)	+	—	—	Died in 10 days.	Died in 10 days.	Died in 13 days.	Died in 10 days.	Died in 10 days.
	(Recovered.)	(Recovered.)	Died in 9 days.	+	(Killed.)	Died in 9 days.	Died in 11 days.	Died in 10 days.	Died in 10 days.
0.0001	—	—	—	—	+	Died in 11 days.	Died in 13 days.	Died in 13 days.	Died in 11 days.
	—	—	—	+	(Recovered.)	Died in 11 days.	(Recovered.)	Died in 10 days.	Died in 11 days.
	—	—	—	—	—	—	Died in 14 days.	Died in 10 days.	Died in 11 days.
0.001	—	—	—	—	—	—	+	Died in 16 days.	Died in 12 days.
	—	—	—	—	—	—	(Recovered.)	Died in 16 days.	Died in 12 days.
	—	—	—	—	—	—	—	+	—
0.01	—	—	—	—	—	—	—	(Recovered.)	Died in 11 days.
	—	—	—	—	—	—	—	Died in 12 days.	Died in 11 days.

	Died in 11 days.	Died in 10 days.
0.1	-	-
1.0	-	-

— indicates that no infection developed; + indicates the development of infection.

not characteristic of anti-*icteroides* serum alone, because various immune serums appear to undergo a similar reduction in strength when introduced into different species of animals. Knorr, for example, found that tetanus antitoxin (horse serum), given to a foreign species (guinea pigs or rabbits), is reduced rapidly and that only about $\frac{1}{100}$



TEXT-FIG. 1. Curve showing the increase in amount of anti-*icteroides* serum (in units) necessary for protection according to the length of time elapsing after administration.

of the quantity originally present in the blood can be demonstrated after 12 to 14 days.⁵ Tizzoni⁶ obtained similar results. Nocard⁷ estimated that the longest period that the tetanus antitoxin remains

⁵ Knorr, A., Das Tetanusgift und seine Beziehungen zum thierischen Organismus. Eine experimentelle Studie über Krankheit und Heilung, *Münch. med. Woch.*, 1898, xlv, 362; Die Entstehung des Tetanusantitoxins im Thierkörper und seine Beziehung zum Tetanusgift, *Fortschr. Med.*, 1897, xv, 657.

⁶ Tizzoni, G., Sull' efficacia dell' antitossina nel trattamento preventivo contro il tetano dopo avvenuto l'infezione, *Gazz. osp.*, 1897, xviii, 1215.

⁷ Nocard, E., Sur la sérothérapie du tétanos; essais de traitement preventif, *Bull. Acad. med.*, 1895, xxxiv, 407.

in the body is about 4 to 6 weeks. Pfeiffer and Friedberger⁸ found that when an anticholera serum derived from the goat is injected into the rabbit it gives rise to an anti-immune substance by which it is neutralized in a comparatively short time. Ransom and Kitashima⁹ showed that homologous tetanus antitoxin, when injected into a normal horse, may remain as long as 80 days; that is, the duration of the passive immunity is almost as long as that of active immunity. On the other hand, Jörgensen and Madsen found that not all homologous immune serums remain for a long period in the body of the injected animal, but that the length of time varies with different animal species.¹⁰ Kraus and his coworkers failed to demonstrate any evidence for the formation of an anti-immune substance in the blood or organs of animals which had received an injection of a heterologous diphtheria antitoxin.¹¹ Perhaps the sudden disappearance of the immune substance after about 10 days in the present series of experiments may be intimately connected with the precipitin formation for the heterologous anti-*icteroides* horse serum. Moreover, the titer of the immune serum is certain to suffer reduction when kept at a temperature of 39°C., even *in vitro*, and may be expected to undergo similar reduction in the blood of a foreign species.

SUMMARY.

Analysis of the records of instances in which non-immune persons contracted yellow fever notwithstanding vaccination shows that the onset of disease occurs soon after vaccination, the longest period being 13 days. Since the average incubation period in yellow fever is 6 days, it seems that infection must have taken place in some instances during the period while protection was developing. These instances

⁸ Pfeiffer, R., and Friedberger, E., Ueber den Verbleib der bacteriolytischen Immunkörper im tierischen Organismus nach der passiven Immunisierung, *Centr. Bakt., 1te Abt., Orig.*, 1904, xxxvii, 131.

⁹ Ransom, F., The conditions which influence the duration of passive immunity, *J. Path. and Bact.*, 1900, vi, 180.

¹⁰ Jörgensen, A., and Madsen, T., The fate of typhoid and cholera agglutinins during active and passive immunisation, *Festskrift ved Indvielsen af Statens Serum-Institut*, 1902, Copenhagen, Paper 6.

¹¹ Kraus, R., and Joachim, J., Zur Frage der passiven Immunisierung, *Wien. klin. Woch.*, 1903, xvi, 1389.

led to a study of the possibility of immediate protection by means of the anti-*icteroides* serum. It had already been shown that the immune serum protects at once against experimental *Leptospira icteroides* infection, but it remained to determine how long the protection would last.

Guinea pigs were given different quantities of the immune serum and subsequently injected, at various intervals, with a virulent strain of *Leptospira icteroides*. Complete protection enduring 5 days was obtained with as minute a quantity of serum as 0.002 cc. per 1,000 gm. of body weight. After 5 days, however, the immune substance rapidly diminished, and to keep the animal protected for as long as 10 days it was necessary to give 100 times as much, or 0.2 cc. For a man weighing 80 kilos, 0.16 cc. (0.002×80) would theoretically be sufficient to protect for at least 5 days, 1.6 cc. for 7 days, and 16 cc. for 10 days. This temporary protection may be a valuable antecedent to that furnished by vaccination, since the final effect of the latter cannot be expected until at least 9 to 10 days have passed.

PURE CULTURES OF LARGE MONONUCLEAR LEUCOCYTES.

By ALEXIS CARREL, M.D., AND ALBERT H. EBELING, M.D.

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PLATES 29 TO 37.

(Received for publication, March 17, 1922.)

I.

INTRODUCTION.

Pure strains of cells are as necessary in the study of physiological problems as pure cultures of microorganisms in bacteriology. If leucocytes and lymphocytes could be kept for a long time in active condition in media of known composition, many fundamental facts concerning their normal function in the organism and their reactions against bacteria and foreign material would be elucidated. In 1920, several attempts were made in this laboratory to obtain a pure culture of large mononuclear leucocytes. Finally, a strain was isolated from the blood of a hen in January, 1921, by one of us, and maintained in active condition for nearly 3 months. Since then, other strains have been cultivated in the same manner. The purpose of this paper is to describe the characteristics of the pure cultures, the morphological changes of the cells, and their response to certain modifications in the composition of the culture medium.

II.

Technique.

1. *Preparation of the Cells.*—The blood of an adult chicken which had fasted for 24 hours was taken in cold paraffined tubes through an oiled cannula or syringe and centrifuged at high speed for 10 minutes. After removal of the plasma, a few drops of diluted embryonic tissue juice were placed at the surface of the buffy coat of

leucocytes. 15 minutes later, the coagulum containing the white cells could be removed and placed in a watch-glass with a small amount of Ringer solution. Generally, the film separated into clumps from which almost all the red cells could be washed away. Small fragments were selected and taken from the fluid with the point of a cataract knife.

2. *Preparation of the Medium.*—Several different media were used. In some experiments, no embryonic tissue juice was used and the medium was composed of plasma alone, or of plasma and Tyrode solution. In other experiments, the medium consisted of two volumes of plasma and one volume of chick embryo juice, or of one volume of plasma, one volume of chick embryo juice, and two volumes of Tyrode solution. Sometimes 20 per cent fibrinogen suspension was substituted for the plasma. In order to prevent the contamination of the white cells by fibroblasts or other cells, the tissue juice was diluted with Tyrode solution and centrifuged for a long time. After the mixture of tissue juice and plasma had been made, the culture medium was examined microscopically and the presence of any contaminating cells could be detected easily. In other cases, the tissue extract, before being used, was kept in cold storage for several weeks until the contaminating cells were dead.

3. *Preparation of the Cultures.*—The culture medium was placed on a cover-glass of mica, and a small fragment of the leucocyte film was embedded in it before coagulation. A certain relation should exist between the number of white blood corpuscles and the volume of the medium. If the leucocytes were too few in number or if the medium was overcrowded by the cells, the result was negative. When the culture was properly prepared, a great many ameboid cells were seen in the medium after 24 hours. Then the original fragment, which still contained a large number of cells, and the invaded area of the coagulum were divided into several parts and washed in Ringer solution. The section of the coagulum had to be made with greater care than in cultures of fibroblasts. If the knife was not very sharp and the edges of the coagulum were crushed or slightly folded, the cells could not migrate into the new medium. They multiplied in the old coagulum and died. After the first passage, the transfer into a fresh medium was made every 48 hours and even every 3rd, 4th,

or 5th day. The cultures were examined while living, and drawings and photographs were made of the cells. Some of them were stained with neutral red in concentrations varying from 1:10,000 to 1:40,000. A few cultures were fixed in warm Ringer solution containing 4 per cent of formalin, and stained with hematoxylin-eosin, Giemsa's stain, eosin and methylene blue, eosin-azure, and azure.

III.

RESULTS.

The first strain of large mononuclear leucocytes was kept in a condition of active life for almost 3 months. Some other strains were used for shorter periods of time.

1. Migration and Multiplication of the Cells.—After 24 hours, the fragment of coagulum containing the white blood corpuscles was surrounded by a very large number of cells which sometimes invaded the entire area of the medium (Fig. 1). The outer zone was made up of small ameboid cells. In fixed and stained cultures they appeared as polymorphonuclear leucocytes which are pseudo-eosinophilic. The inner zone consisted chiefly of larger ameboid cells. Some of them were very large and more or less elongated and branched, with reticular pseudopods. The others were smaller, more rounded in form, and emitted active filiform or lobar pseudopods. In the preparations stained with eosin-azure, these cells contained a darkly stained nucleus and some showed blue granules in their cytoplasm. They were lymphocytes and large mononuclear leucocytes. Some polymorphonuclear cells were also present in the inner zone. After 24 hours, fragments of coagulum containing the ameboid cells were extirpated from the cultures and placed in a new medium. The cells migrated almost immediately from the old into the new coagulum. They consisted of polymorphonuclear leucocytes, lymphocytes, and large mononuclears, but the number of polymorphonuclear leucocytes had markedly decreased (Fig. 2). If the cells were too closely packed in the old coagulum, their migration into the new medium was slow and death often occurred. After the third or fourth passage, no granular leucocytes remained in the cultures, which were composed merely of lymphocytes and large mononuclear cells. Later, the lymphocytes disappeared also (Fig. 3).

The activity of the cultures was irregular. The migration of the cells (Figs. 4 to 6) from the edges of the old coagulum started from the 1st to the 4th day. When a sharp section of the coagulum was made in the zone of the migrating cells, the medium was invaded sooner. During the first 24 hours, the cells multiplied in the old coagulum and accumulated in large numbers at the periphery where they sometimes formed masses of amorphous material. Later, a few ameoboid cells grew from the edges (Figs. 4 to 6) and more or less suddenly toward the 3rd day a large number of them invaded the new medium. In other cases, the invasion of the medium began on the 1st day. The cells were usually disposed in chains which followed almost parallel paths into the new medium on several different planes, but every chain was laterally isolated from the others. When the growing masses were very dense and the cells in contact, the cultures generally died. Often chains of independent cells migrated far into the new medium. This phenomenon occurred generally after 2, 3, or 4 days, according to the technique used, the composition of the medium, and the previous activity of the cells. The multiplication of the cells was slow. However, a few mitotic figures were observed (Figs. 7 and 8). A normal culture was composed of a thick crown of elongated and branched cells, while the central part of the coagulum was practically free of cells. When the old coagulum contracted, or when the crushing or folding of its edges prevented migration in a measure, living cells and apparently dead material were observed in the center of the culture.

The general appearance of the cultures was strikingly different from that of a fibroblast culture. The cells had no tendency to form a tissue (Figs. 2 and 3), but always remained isolated. If they happened to be packed together, they died. When colonies of leucocytes were grown in Gabritschewski dishes, their edges showed no tendency to unite (Fig. 9).

The large mononuclear leucocytes invading the culture medium were almost uniform in shape, elongated or branched, and of irregular form. Their posterior end was generally rounded, while the anterior end emitted very active reticular pseudopods (Figs. 4 to 6). The nucleus was darkly stained by azure while the cytoplasm was clear. In the living cultures stained with neutral red, the cells showed a

segregation apparatus consisting of spherical bodies, often as large and even larger than the nucleus. After each passage, chains of cells of the same type, more or less densely packed, again invaded the medium. But under the influence of many technical conditions, and especially the composition of the culture medium, their rate of proliferation was liable to decrease. The two first strains which were isolated in January, 1921, lost their activity at the beginning of April and died soon afterwards. Another is still active after 3 months of life *in vitro*.

2. *Differentiation of the Large Mononuclear Leucocytes.*—In actively growing cultures, the appearance of the cells remained uniform. The strains were composed of ameboid cells, with more or less irregular darkly stained nuclei, and lobar, filiform, or reticular pseudopods (Figs. 5 and 6) which wandered from the edges of the old coagulum into the new medium. However, important morphological changes sometimes occurred. They were observed for the first time in December, 1920. Leucocytes had been taken from the blood of a hen and cultivated in homogenic plasma without the addition of any tissue juice. The coagulum was soon invaded by a large number of ameboid cells, which wandered through it for 2 days. Some of the cells agglutinated in clumps. At the periphery of, or within the clumps, a few cells modified their shape. The pseudopods disappeared and were replaced by sharp processes. On the 3rd day, fusiform and stellate cells resembling fibroblasts were seen in some of the clumps. There was no possible contamination of the culture by fibroblasts from another source because the cells had been taken from the blood and no tissue juice had been added to the plasma composing the medium.

The same phenomenon (Fig. 10 *a* and *b*) was observed subsequently in many cultures. It occurred generally when the migration and the multiplication of the lymphocytes were not very active. The transformation of the cells started in the old coagulum and its origin was multicentral. The cells which showed the first evidence of a change were the elongated lymphocytes with clear cytoplasm and reticular pseudopods. The central end of the cell lost its pseudopods and became a pointed and immobile process, while the peripheral end was still actively ameboid. These transition forms were apparently half fibroblasts and half ameboid cells. Sometimes the nucleus of an

ameboid cell lost its character of being darkly stained by azure, and became pale and oblong with two nucleoli like that of a typical fibroblast. Similar changes occurred in polygonal and branched cells which showed simultaneously fixed processes and reticular pseudopods. At the same time, typical macrophages (Fig. 11*b*) were seen at the periphery of the culture and sometimes in the immediate vicinity of the transition forms and the fibroblasts (Figs. 10*b* and 11*a*). The fibroblasts appeared often near the amorphous masses resulting from the accumulation of lymphocytes on the edges of the old coagulum. Pointed processes seemed to grow from this apparently necrotic material, and after a few hours, fusiform cells with slender processes could be seen. In a few cultures, the fibroblasts migrated from the old coagulum into the new medium where ameboid cells were still wandering (Fig. 10*b*). They were spindle-shaped or stellate cells with an outline not sharply defined, anastomotic processes, pale homogeneous cytoplasm, large oval nuclei, and generally two nucleoli (Fig. 12*a* to *d*). Of four cells united in a chain, two resembled spindle-shaped fibroblasts, the third was half fibroblast and half ameboid, and the fourth was a large mononuclear leucocyte. In this case it seemed that a chain of large mononuclears was being changed into fibroblasts, the mutation beginning in the central part of the coagulum. In other cultures, several fibroblasts were united by long processes in a reticulum.

Cultures which contained large mononuclears, transition forms, and cells resembling fibroblasts were stained with a weak solution of neutral red. The ameboid cells displayed large red granules, which sometimes filled the cytoplasm almost completely. Among the transition cells, some showed a large segregation apparatus and others small red granules. The fibroblastic forms presented very much finer granules, and some were completely free of them. In the latter case, the nucleus was oblong and clear, contained two nucleoli, and looked like the nucleus of a fibroblast. At the same time that their appearance was modified, the behavior of the cells changed. Instead of remaining isolated or forming a chain, they became able to unite sidewise with other cells and showed a tendency to form a tissue, while lymphocytes lived like isolated elements. The cells were acquiring not only a new morphological appearance but also the

property of associating with homologous cells in the manner of fibroblasts.

3. *Action of Embryonic Tissue Juice and Serum on the Activity of the Cells.*—It seemed probable that lymphocytes and large mononuclear leucocytes would respond to the presence of embryonic tissue juice and serum in the medium as do fibroblasts. But the sensitiveness of their reaction had to be investigated.

(a) In the experiments in which the action of embryonic tissue juice was tested, two kinds of media were used. The first was composed of 33.3 per cent normal plasma and 66.6 per cent of a mixture in different concentrations of embryonic tissue juice and Tyrode solution. The second was made of fibrinogen suspension, serum, Tyrode solution, and embryonic tissue juice. Large mononuclears in a condition of low activity at the third or fourth passage were cultivated in media containing 2.5, 33.3, and 66.6 per cent tissue juice. In 2.5 per cent tissue juice, very little or no migration took place and death occurred after one or two passages. On the contrary, the medium containing 33.3 per cent tissue juice was invaded by a large number of lymphocytes. In 66.6 per cent tissue juice, the migration was still greater, but the cells died after a short time, possibly because they were too closely packed. Similar experiments were made with a medium composed of 20 per cent fibrinogen suspension and 10 per cent serum, the presence of serum being necessary to obtain a firm clot which would not dissolve secondarily. The action of 2.5 and 40 per cent embryonic tissue juice was compared. The number of migrating cells was very much larger in the medium containing 40 per cent tissue juice, but they died sooner than in the 2.5 per cent medium. It was evident that the cells were very sensitive to the influence of the embryonic tissue juice, and that its optimum concentration in the medium was about 30 per cent.

(b) The action of homologous serum was studied in media composed of 20 per cent fibrinogen suspension, 5 per cent tissue extract, and varied concentrations of Tyrode solution and serum. The cells were obtained from cultures at the third and fourth passages and cultivated in media containing 10 and 50 per cent serum. In the first medium, no migration took place. The cells located in the old coagulum became round, and very few showed active ameboid

movements. They died after the first passage. On the contrary, in the second medium the cells located in the old coagulum retained their activity and a large number migrated into the new medium. After they were transferred to fresh medium, they perpetuated themselves for several generations. Similar results were obtained in media containing 70 and 10 per cent serum. There was no doubt that serum had a marked inhibiting influence on the migration of lymphocytes and large mononuclear leucocytes.

IV.

DISCUSSION.

The cells growing in pure cultures were large mononuclear leucocytes of Ehrlich (endothelial leucocytes, monocytes of Naegeli, blood histiocytes of Aschoff and Kiyono, or hemomacrophages of Metchnikoff). The pseudo-eosinophilic and eosinophilic leucocytes were never observed to be present after five or six passages. The lymphocytes, that is the small and medium sized mononuclear cells of Ehrlich, were seen in large numbers during the 1st week and disappeared afterwards spontaneously. Possibly they were transformed into larger forms, as Maximoff¹ observed in his cultures of lymphoid tissue. Then the cultures were composed exclusively of large mononuclear leucocytes. These cells maintained their activity *in vitro* for nearly 3 months. However, their rate of multiplication was slower than that of fibroblasts. The medium was probably not the optimum one. ✓It may also be supposed that they cannot grow in solid medium in dense masses as fibroblasts do. When they were packed in the coagulum, they usually died after a short time. The actively growing cultures showed a large number of chains of ameboid cells radiating from the original fragment through the medium. But the chains were not in contact laterally. It seemed that the cells created around themselves a field unfavorable to homologous cells. They always remained isolated, while fibroblasts and epithelial cells in pure cultures formed a tissue. The colonies of lymphocytes and fibroblasts when in contact assumed a strikingly different appearance. The edges of fibroblast colonies had a marked tendency to unite.

✓ ¹ Maximoff, A., *Compt. rend. Soc. biol.*, 1917, lxxx, 225.

In some cultures, the fibroblasts of a colony seemed to be attracted by the fibroblasts of the neighboring colony and a group of several colonies soon formed a continuous layer of connective tissue (Fig. 13 a). On the contrary, the peripheral edges of the leucocyte colonies never coalesced, even when growing in close contact (Fig. 13 b).

The large lymphocytes were more sensitive to all influences than the fibroblasts. The slightest error in technique brought about the death of the cultures. This peculiarity increased the difficulties of the experiments very much, because marked differences of growth were observed which were due merely to technical errors. However, the cells responded readily to physical and chemical modifications of the composition of the medium by changes in the rate of migration and in their morphology. As cells cultivated *in vitro* respond to an antigen by the production of an antibody,² the use of pure strains of macrophages may, therefore, become of interest in the study of immunity.

The large mononuclears became transformed into cells which assumed the appearance of fibroblasts. These cells were spindle-shaped or stellate, with long and slender processes uniting sometimes in a reticulum with those of other cells. When stained vitally with neutral red, they could be distinguished from the large mononuclears present in the same culture by the neutral red reaction of Evans.³ The nucleus was similar to that of the fibroblasts. They had also acquired a tendency to tissue formation which lymphocytes do not possess. Besides the typical cells, there were many transition forms characterized by a nucleus less darkly stained and more oval, by the progressive substitution of sharp processes for the pseudopods, and by a decrease in the size of the segregation apparatus. The macrophages had evidently differentiated into fibroblasts. It is probable that Maximoff observed the beginning of a similar transformation in his cultures of lymphoid tissue.¹ He mentions that the large lymphocytes cultivated in hypotonic plasma and bone marrow extract underwent a modification. Their cytoplasm was larger and less basophilic, while the nucleus became paler.

² Carrel, A., and Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 287.

✓ ³ Evans, H. M., and Scott, K. J., *Carnegie Institution of Washington, Pub. No. 273, Contributions to Embryology*, 1921, x, 3.

The differentiation of large mononuclears into fibroblasts is merely the confirmation of previous observations. Long ago, Renaut⁴ found that young connective tissue formed inside uterine cysts, completely lined with epithelial cells, and thought it derived from ameboid cells which had migrated through the epithelium. He considered that fixed connective tissue cells, endothelial cells, clasmotocytes, chromoblasts, vacuolar cells, and osteoblasts originated from an indifferent lymph cell. The transformation *in vitro* of connective tissue macrophages into fibroblasts came as a partial confirmation of this view. In 1912, pure cultures of ameboid cells from connective tissue became transformed into fibroblasts.⁵ Cultures of peritoneal macrophages were observed by Maximoff⁶ to undergo differentiation and to yield colonies of fibroblasts. Maximoff^{6,7} never observed the transformation of polyblasts into fibroblasts, probably for technical reasons. Foot,⁸ in cultivating white blood corpuscles of chicks, saw no real fibroblasts in the preparations. But in cultures of leucemic blood, Awrrow and Timofejewskij⁹ found that the lymphocytes, in multiplying and undergoing differentiation, were converted into various forms—macrophages, giant cells, and even spindle-shaped and stellate connective tissue cells.

It is well known that the genetic relationship between the types of connective tissue cells is very close. Sabin¹⁰ has shown lately that clasmotocytes and monocytes, that is large mononuclear leucocytes, are identical in origin. Their differentiation into fibroblasts is, therefore, a phenomenon which could easily be expected and was already considered as certain. Policard and Desplas¹¹ consider that fibroblasts derive from lymphocytes in granulating wounds. According to Dubreuil,¹² the large mononuclear leucocytes wander into and become fixed in the connective tissue, grow larger, and transform

✓ ⁴ Renaut, J., *Traité d'histologie pratique*, Paris, 1893, i, pt. 2, 968.

⁵ Carrel, A., *J. Exp. Med.*, 1912, xvi, 165.

⁶ Maximoff, A., *Arch. Russ. Anat., Hist. et Embryol.*, 1916, i, 105.

✓ ⁷ Maximoff, A., *Compt. rend. Soc. biol.*, 1917, lxxx, 237.

⁸ Foot, N. C., *J. Exp. Med.*, 1913, xvii, 43.

✓ ⁹ Awrrow, P. P., and Timofejewskij, A. D., *Virchows Arch. path. Anat.*, 1914, ccxvi, 184.

✓ ¹⁰ Sabin, F. R., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 314.

✓ ¹¹ Policard, A., and Desplas, B., *Compt. rend. Soc. biol.*, 1917, lxxx, 745.

✓ ¹² Dubreuil, G., *Arch. anat. micr.*, 1913-14, xv, 53.

themselves into clasmatoocytes or rhagiocrins. Then their segregation apparatus decreases, and they become fixed connective tissue cells. The differentiation *in vitro* of lymphocytes into large mononuclears observed by Maximoff,⁷ and of the large mononuclears into fibroblasts recorded by us, is a confirmation of the ideas of Renaut and his school,^{4,12} who consider the lymphocyte as the origin of all connective tissue cells. The transformation of large mononuclears into fibroblasts *in vitro* has more significance than the mere final demonstration of a phenomenon which was practically known. Thus, it shows that cell differentiation may occur *in vitro* under conditions which can easily be controlled. There is, therefore, a basis for the hope that the immediate cause of the change will be discovered.

The pure cultures of large lymphocytes were found to respond more readily than fibroblasts to the presence of embryonic tissue juice or serum in the medium. Lymphocytes and polymorphonuclear leucocytes displayed a similar sensitiveness to the same factors. This means that leucocytes are submitted to the influence of the two classes of substances which we found to be instrumental in increasing and inhibiting the rate of cell multiplication. The activating substances,¹³ as is well known, are contained in embryonic tissues and also in muscle, epithelial cells, and white blood corpuscles of adult animals, in the aqueous extracts of these tissues, and probably in their secretions. Their presence in a culture medium greatly increases the activity of fibroblasts,¹⁴ epithelial cells, and also leucocytes. If leucocytes could be activated *in vivo* by tissue juices as much as *in vitro*, important consequences should follow. "The humors in which tissue juices or cell secretions are set free would acquire the power of increasing the activity of leucocytes, lymphocytes, and fibroblasts. Aqueous extract of inflamed connective tissue, and peritoneal exudates, containing white blood corpuscles, have been found to possess such a power.¹⁵ "The death of groups of cells within a tissue would also set free their juices and stimulate the activity of leucocytes and lymphocytes. Should white cells secrete more freely or undergo leucolysis, the substances which were set free would stimulate the

¹³ Carrel, A., *J. Exp. Med.*, 1913, xvii, 14.

¹⁴ Carrel, A., *J. Exp. Med.*, 1913, xviii, 287. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317.

¹⁵ Carrel, A., unpublished experiments.

activity and multiplication of the macrophages and other cells. By this mechanism, the destruction of a number of leucocytes within the organism should automatically be followed by an increase in activity and by the multiplication of the remaining cells.

It is known that blood serum possesses the power of inhibiting the multiplication of homologous fibroblasts,¹⁶ and that leucocytes respond still more readily than fibroblasts to the retarding influence of serum *in vitro*. Very likely, serum possesses a similar action *in vivo* because the number of leucocytes would increase indefinitely if serum were not endowed with this property. As a consequence of the sensitiveness of leucocytes to serum, these cells must be more active in fluids containing serum proteins under a lower concentration. The relative activity and migration of leucocytes in interstitial lymph, inflammatory exudates, and blood may depend in some measure on this property. It is also probable that the increase of the growth-inhibiting action of serum in the course of life determines a decrease in the activity of the white cells and modifies their secretions. Possibly this is one of the mechanisms by which the profound changes brought about by age in blood serum may be related to certain diseases of the period of senescence, such as cancer. On the other hand, it must be remembered that within the organism the leucocytes are placed in a very complex medium, and that their response to the action of serum and tissue juices may be modified and even prevented by other factors.

V.

CONCLUSIONS.

1. Pure strains of mononuclear leucocytes were isolated from the blood of adult chickens and kept in active condition for nearly 3 months.
2. The cultures were composed of large mononuclear leucocytes which migrated and proliferated *in vitro* at a slower rate than fibroblasts. The cells had no tendency to form a tissue, as do fibroblasts and epithelial cells. They were much less resistant than fibroblasts.
3. Differentiation of the large mononuclears into cells assuming the appearance of fibroblasts took place under certain conditions.
4. The activity of the large mononuclears was increased by embryonic tissue juice and inhibited by homologous serum.

¹⁶ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.

EXPLANATION OF PLATES.

PLATE 29.

FIG. 1. 24 hour culture of leucocytes taken from the blood. In the lower left corner is seen part of the original mass of cells. The coagulum is invaded by a large number of cells. The inner zone is composed chiefly of lymphocytes and mononuclear leucocytes and the outer zone of polymorphonuclear leucocytes. The intermediate zone which appears as a darker ring is occupied by large mononuclear ameboid cells. $\times 62$.

PLATE 30.

FIG. 2. Second passage. The culture is still composed of mononuclear and polymorphonuclear leucocytes. The inner zone contains irregularly shaped, more or less elongated, ameboid cells, and the outer zone polymorphonuclear leucocytes, the number of which has decreased considerably. $\times 133$.

PLATE 31.

FIG. 3. Twelfth passage. Pure strain of large mononuclear leucocytes. The polymorphonuclear leucocytes have completely disappeared. $\times 240$.

PLATE 32.

FIGS. 4 to 6. Large mononuclear cells, with active pseudopods. $\times 1,000$.

FIGS. 7 and 8. Mitotic figures in a pure culture of large mononuclears. $\times 1,000$.

PLATE 33.

FIG. 9. Colonies of leucocytes in a Gabritschewski dish, showing their lack of coalescence. $\times 2.5$.

PLATE 34.

FIG. 10. Culture 26330, eighth passage. (a) Pure strain of mononuclear leucocytes undergoing differentiation. There are a number of mitoses scattered throughout the preparation. $\times 100$. (b) Higher magnification of the same culture. $\times 250$.

PLATE 35.

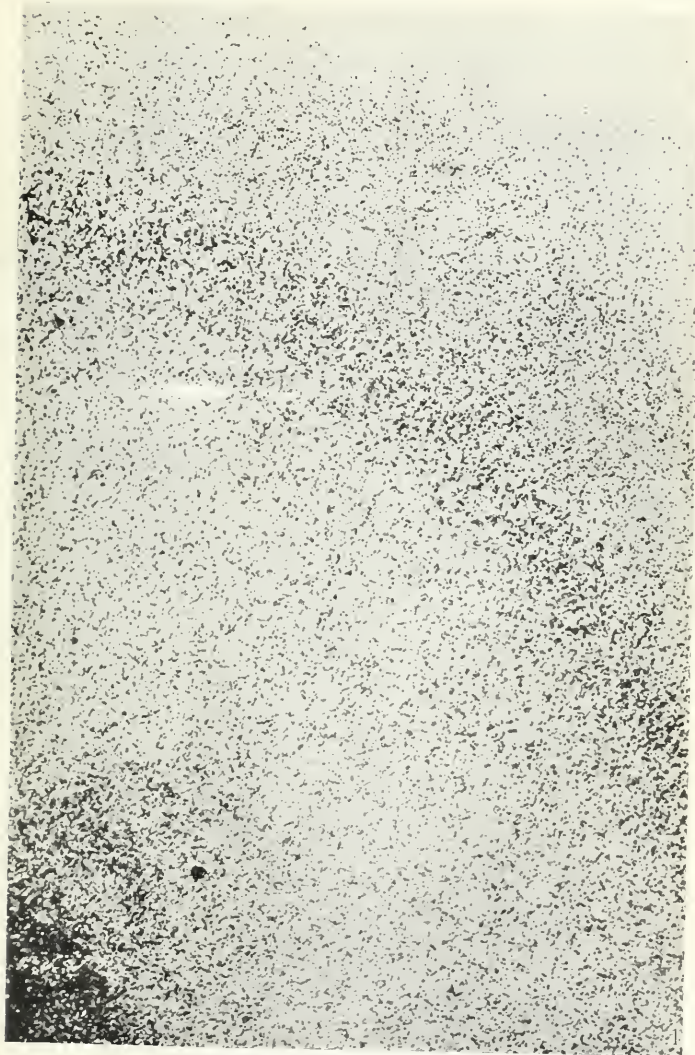
FIG. 11. Culture 26330, eighth passage. Higher magnification of two neighboring cells from the culture shown in Fig. 10. (a) Large mononuclear assuming the appearance of a fibroblast. $\times 1,000$. (b) Large mononuclear, actively ameboid. $\times 1,000$.

PLATE 36.

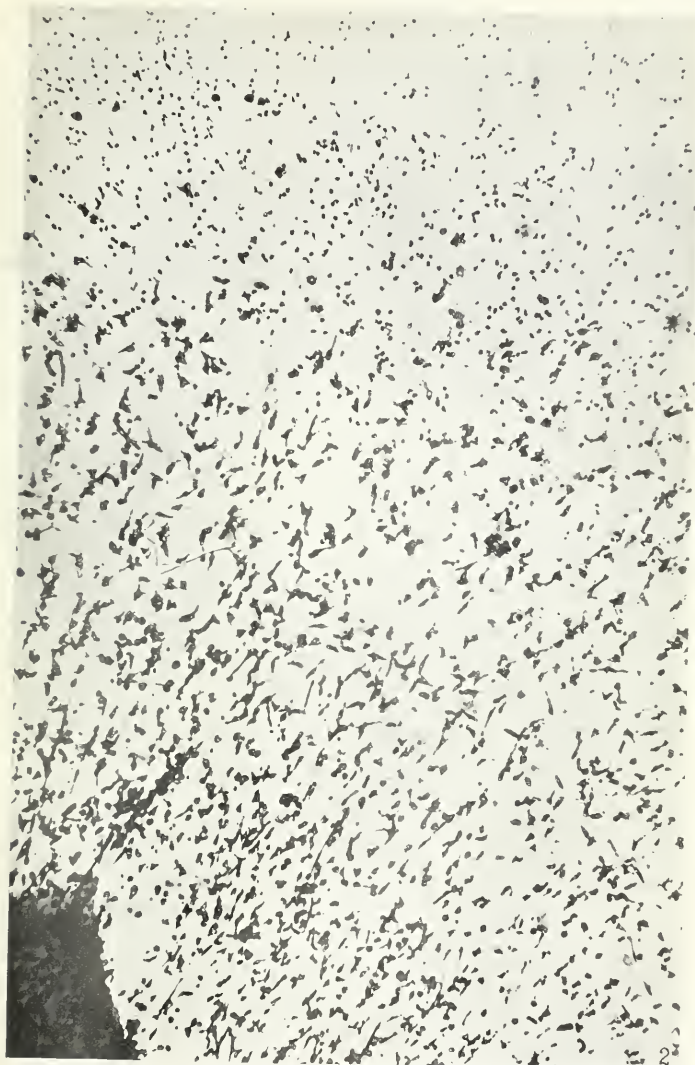
FIG. 12. (a to d) Large mononuclear leucocytes transformed into spindle-shaped and stellate forms. $\times 1,000$.

PLATE 37.

FIG. 13. Semidiagrammatic drawing of colonies of (a) fibroblasts and (b) leucocytes. The colonies of fibroblasts have united at their edges.



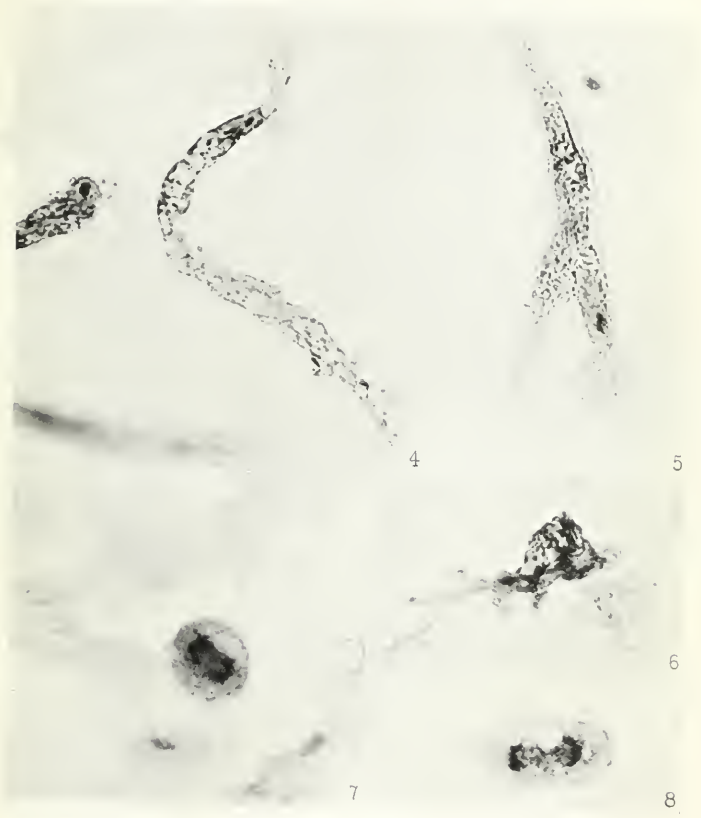
(Carrel and Ebeling: Large mononuclear leucocytes.)



(Carrel and Ebeling: Large mononuclear leucocytes.)

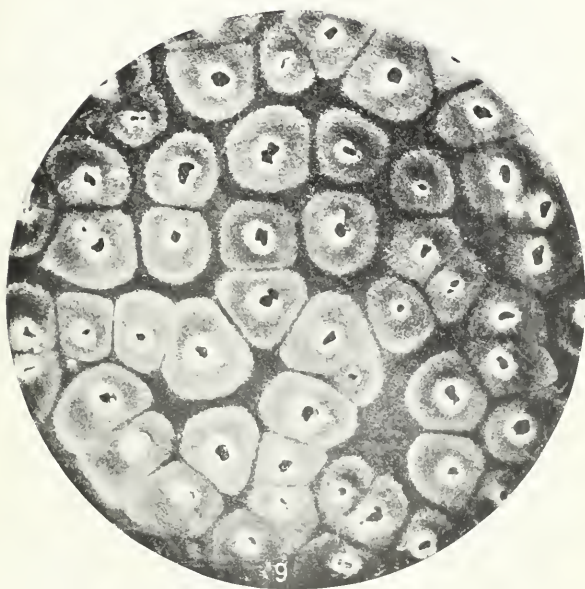


(Carrel and Ebeling: Large mononuclear leucocytes.)



(Carrel and Ebeling: Large mononuclear leucocytes.)

378



(Carrel and Ebeling: Large mononuclear leucocytes.)



(Carrel and Ebeling: Large mononuclear leucocytes.)

218⁷

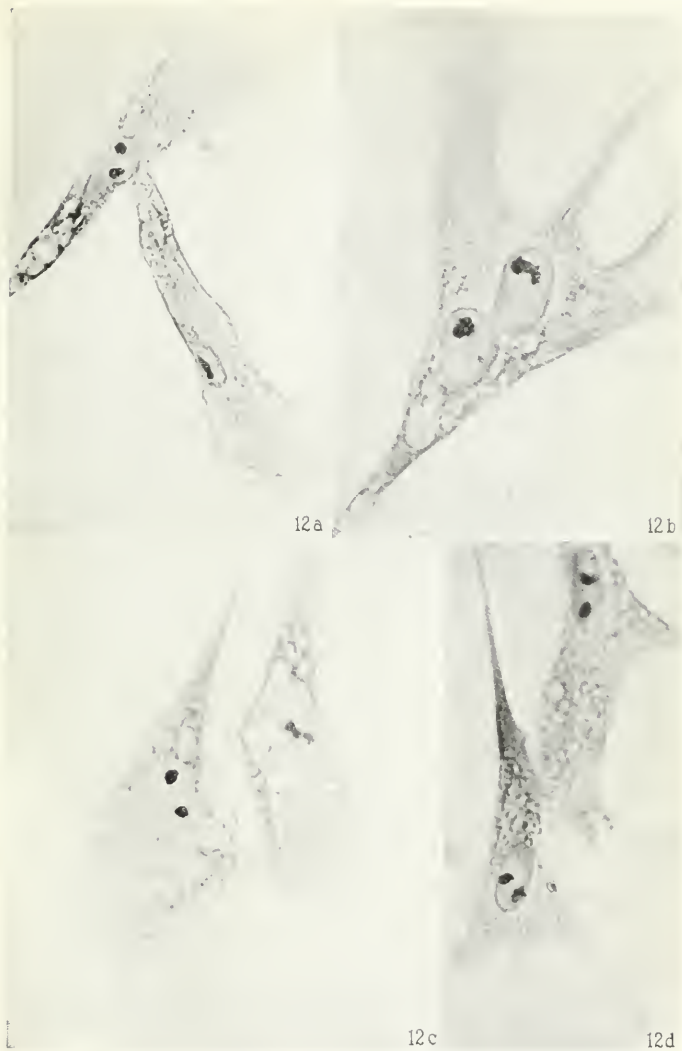


11a

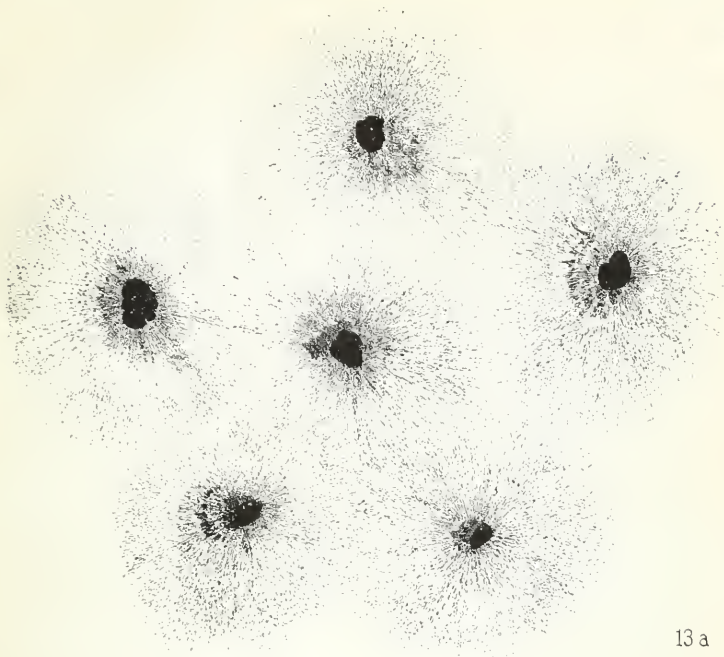


11b

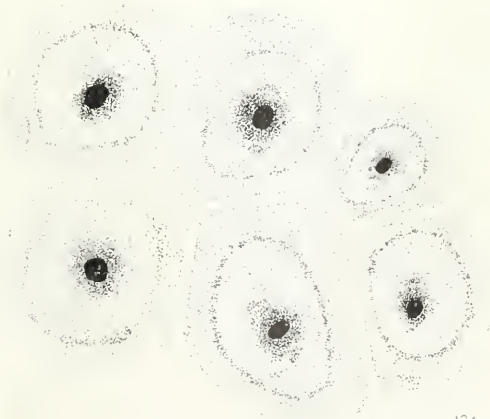
(Carrel and Ebeling: Large mononuclear leucocytes.)



(Carrel and Ebeling: Large mononuclear leucocytes.)



13 a



13 b

(Carrel and Ebeling: Large mononuclear leucocytes.)

A PURE STRAIN OF CARTILAGE CELLS IN VITRO.

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PLATES 38 TO 40.

(Received for publication, April 4, 1922.)

I.

INTRODUCTION.

No pure strain of cartilage has so far been isolated. However, the importance of working with pure cultures of the various types of tissue cells has been shown.¹ At present, only one method of obtaining a pure strain of a given type is known; namely, the isolation of tissues for cultivation from portions of the organism where they may be obtained free from any other tissue elements. In the eye of the chick embryo, we found an anatomical *locus electus* for obtaining pure cartilage. The sclera of the posterior hemisphere of the bulbus oculi in most birds and fish has a pars cartilago scleræ, and sometimes even a pars ossea. In sectioning the eye, it was noticed that free dissection of the cartilage could be made.

II.

EXPERIMENTAL.

1. *Isolation of the Cartilage.*—When the excised eye-bulb of the chick embryo is placed on its cornea, one may easily push aside the dark pigmented membrana nictitans with a small knife and an iris forceps; just below is seen a thin, white, semitransparent membrane. This is the cartilage. With the small forceps it may be lifted out. Immediately upon its removal, it retracts as does a drop of mucus, but when placed in a little Ringer solution, it spreads out as a thin membrane. In other respects it is not at all like mucus, but is very fragile and disintegrates easily.

¹ Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367.

2. *Technique and Description of the Cultures.*—The culture medium is the same as that generally used for connective tissue cells and epithelium, consisting of equal volumes of adult chicken plasma and juice from chick embryos. The fragments of cartilage are placed on the surface of the coagulated plasma medium. Usually the growth does not begin in a promising way, but after three to five passages it becomes very active. If the piece of cartilage is embedded in the medium, as is done in cultivating fibroblasts no growth seems to take place. The fragment of cartilage remains apparently unchanged in shape in the same medium for many days, but by measuring the area of cartilage the day the culture is made and again after 3 or 4 days, or even longer, it is found to have increased in size. It is not certain whether this increase is due to a loosening of the tissue or to a veritable increase in the number of cells. When an attempt was made to transfer such a piece of cartilage into a new medium, after it had been embedded in the coagulum for 2 or 3 days, it proved impossible. The cartilage was so fragile that the slightest touch with the knife shattered it into innumerable pieces. If one is fortunate enough to secure a small piece on the knife and put it in Ringer solution, it spreads out very quickly, closely resembling a thin membrane of paraffin in water and being just as fragile. When it is placed in the new medium and examined under the microscope, thousands of aggregates and single cells are seen spread all over the medium. This picture is strikingly like that of a culture of yeast, especially because the cells are so small and apparently spherical. Therefore, no growth seems to take place from these colonies of cells after they are embedded in the coagulum.

An attempt was then made to place one of the small pieces of cartilage on the free surface of the clotted medium. As soon as this was done, the cells began migrating and progressively became more active and formed huge cultures. Cultivated in this way, the growth was very extensive and the cultures could be multiplied. These new cells grew in some ways like epithelium, forming thin, delicate membranes, while the individual cells were in close contact. The cultures were no longer fragile but coherent and easy to extirpate and transfer to a fresh medium.

When first examined, it seemed as if a new kind of tissue had formed which, in a measure, was true. The amorphous hyaline substance

had disappeared, and the cartilage cells grew and proliferated very actively side by side, without forming any of the hyaline substance. In order to study the details of this transformation, fresh cartilage was cultivated, embedded in the clot as well as on the free surface, and the cultures were fixed and stained in different stages. It was observed that the amorphous substance was loosened and fell to pieces, and the cartilage cells became free. The space around the cells could be seen to increase in size, and finally the hyaline substance liquefied completely (Fig. 1). The cartilage cells themselves were rather small, and the cytoplasm as well as the nucleus stained deeply. When the cartilage was embedded in the clot, the cells seemed unable to grow or to develop, unless they were in very close contact. Those cells which had been cultivated for some time in the clot were all very small. Nucleus and cytoplasm, the latter often very sparsely represented, were deeply stained (Figs. 2 and 9). The cells were about the size of small lymphocytes and the resemblance was rather striking.

From the surface-cultivated cartilage, the same process of breaking down the hyaline substance took place and a different type of cells migrated. All the transition forms could be studied here, from the small, lymphocyte-like cartilage cell to the much larger cell which finally characterized the active cartilage cultures. The same phenomenon could be observed as in Fig. 1, and the free cells gained in size rather quickly; *i.e.*, their cytoplasm was spread out (Figs. 3 and 4). Some resembled the cells in Fig. 5, having a deeply stained nucleus with the chromatin located at the periphery, and cytoplasm unstained and nearly as clear as glass. After further cultivation, the cells became spindle-shaped and the cytoplasm increased more and more, the cells finally appearing as in Figs. 6 and 10. The cytoplasm often contained large vacuoles. The nucleus was spherical and usually had one large nucleolus. The nucleus itself generally stained less deeply than the cytoplasm. The cells grew in close contact, forming thin membranes.

If, in this stage, the culture is transferred to the middle of the coagulum, it grows well and does not revert to the type of small cells.

III.

DISCUSSION.

Renaut and Dubreuil² came to the conclusion that the embryonic connective tissue originated from the lymphocytes. The various functions of the connective tissue determine the peculiarities of the amorphous substances excreted. In cartilage the collagen substance (connective tissue fibrillæ) is replaced by the hyaline substance. The transformation of lymphocytes into large mononuclears and polyblasts was observed by Maximoff,³ and of large mononuclear lymphocytes into fibroblasts by Carrel and Ebeling.⁴

In our experiments, it was observed that a minute, lymphocyte-like cell type migrated out from the cartilage and became rapidly transformed into cells many times larger than the original ones. They did not produce any hyaline substance. When the size of the cells increased, great morphological and physiological changes occurred in them. The cytoplasm of the small cell type was very sparse, and the nucleus had a granular chromatin arranged in its periphery. Both nucleus and cytoplasm stained very deeply with methylene blue and azure blue. On the contrary, the cells which grew out on the surface of the coagulum were large and their cytoplasm was often filled with vacuoles. The nucleus generally had one large nucleolus and stained less deeply than the cytoplasm.

The segregation apparatus of Renaut when vitally stained with neutral red appears to be very similar to that of fibroblasts (Fig. 7). It was found that the initial growth of cartilage only took place on the free surface of the coagulum. When embedded in the coagulum, the cells were unable to adhere and no growth took place.

The strain of cartilage now under cultivation is more than 3 months old, grows very actively, and can be made to multiply. During this time it has been cultivated on the free surface of the coagulum which, at present, we find to be the best method for obtaining large cultures. Of all tissues hitherto cultivated *in vitro*, it resembles epithelium most, because of its characteristic growth in membranes (Fig. 8). The

² Renaut, J., and Dubreuil, G., *Compt. rend. Soc. biol.*, 1910, lxxviii, 599.

³ Maximoff, A., *Arch. Russ. Anat., Hist. et Embryol.*, 1916, i, 105.

⁴ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 365.

cartilage liquefies the medium much more than epithelium and connective tissue cells do.

IV.

CONCLUSIONS.

1. A strain of cartilage cells, obtained from the pars cartilago scleræ of the eye of chick embryos, has been cultivated for more than 3 months *in vitro*.

2. The initial growth of the cartilage was possible only on the free surface of the coagulum.

3. The hyaline substance disappeared during cultivation *in vitro*. The succeeding stages of a transformation from small, lymphocyte-like cells into large, spindle-shaped cells were observed. The cartilage cells were spindle-shaped and grew in close contact, forming thin membranes. In surface-grown cartilage cells, the nucleus, usually containing one large nucleolus, stained less deeply than the cytoplasm.

4. The rate of growth of cartilage was slower than that of fibroblasts and epithelium. After cultivation on the surface of the coagulum, the cartilage cells could multiply even when embedded in the coagulum. But their growth was less extensive and uniform.

EXPLANATION OF PLATES.

PLATE 38.

FIG. 1. Cartilage after 48 hours incubation in plasma medium. The amorphous substances are broken up. The cartilage cells contain relatively little cytoplasm and the nucleus and cytoplasm are stained very deeply. Stained with azure blue.

FIG. 2. Types of isolated cells of cartilage from 2 day old cultures.

FIG. 3. Types of cartilage cells after escape from the amorphous substances.

FIG. 4. Types of cartilage cells cultivated on the surface of the medium.

FIG. 5. Cell types of cartilage found in cultures of fresh cartilage after 48 hours incubation. Nuclei deeply stained, with the granular chromatin arranged in the periphery. The cytoplasm is unstained and clear as glass.

FIG. 6. Cartilage cells growing on the surface of the medium.

PLATE 39.

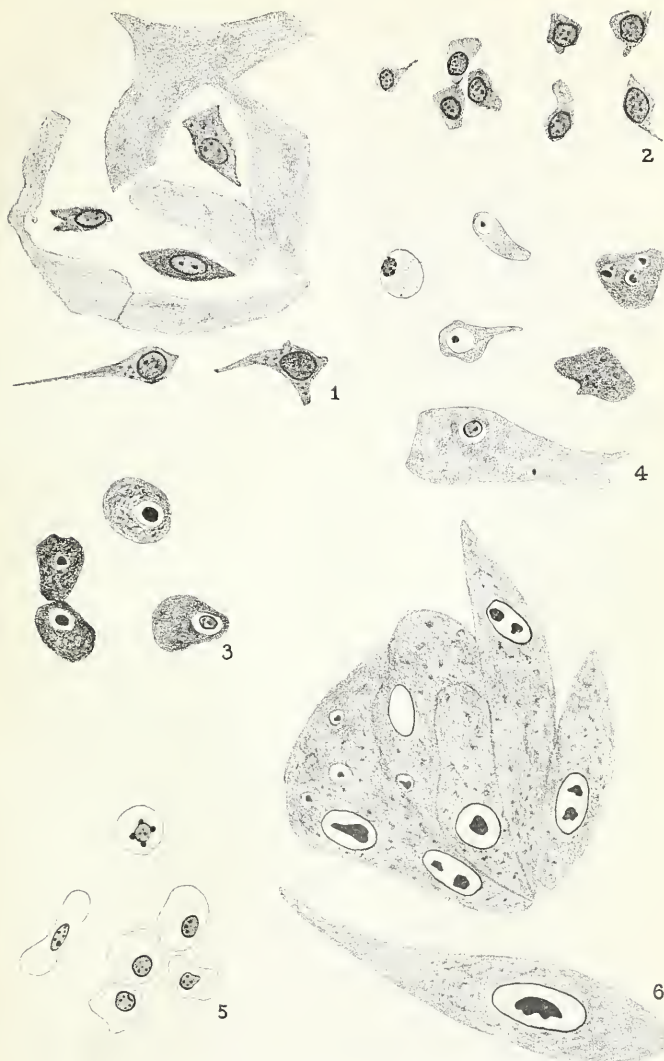
FIG. 7. The segregation apparatus of Renaut, vitally stained with neutral red. The cultures are 6 weeks old; 24 hours incubation.

FIG. 8. 2 month old culture of cartilage cells after 48 hours incubation. Stained with azure blue. $\times 60$.

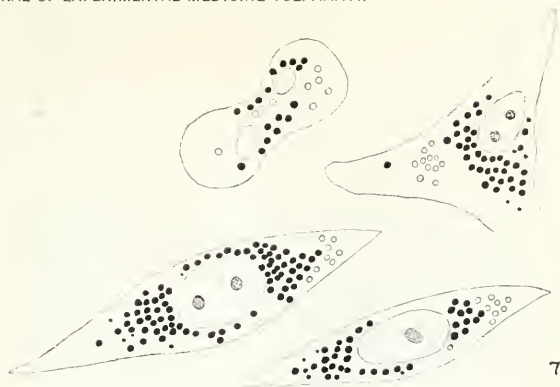
PLATE 40.

FIG. 9. Fresh cartilage cultivated on the surface of the plasma clot, showing the small types of cells just migrating on the medium. Stained with azure blue. $\times 1,000$.

FIG. 10. Experiment 1634. Mitotic figures in cartilage cells. Stained with azure blue. $\times 1,000$.



(Fischer: Pure strain of cartilage cells *in vitro*.)



(Fischer: Pure strain of cartilage cells *in vitro*.)



(Fischer: Pure strain of cartilage cells *in vitro*.)

GROWTH-PROMOTING FUNCTION OF LEUCOCYTES.

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(Received for publication, April 10, 1922.)

It is well known that the tissues of an adult animal remain capable of resuming the activity characteristic of youth. Even in extreme old age, wounds always heal, although cicatrization may require the formation of a large amount of new tissues. We are as yet ignorant of the mechanisms which cause cells at rest in a growth-inhibiting medium, such as the humors of an old animal, to proliferate again. The activity of a cell at a given instant is function of its activity at the preceding instant and of the concentration of certain substances in its medium.¹ Therefore, it is probable that tissues which have been in a resting condition for several years cannot grow again unless they receive the food material required by the cells for their multiplication. We know that certain substances contained in embryonic juice are endowed with the remarkable property of greatly activating the rate of cell proliferation *in vitro*.² Possibly such growth-promoting substances must be supplied to adult tissues when they cicatrize or regenerate. One of the sources of these substances may be the leucocytes which, remaining in the embryonic stage of development during the entire life of the organism, probably contain the growth-activating substances characteristic of embryonic tissues. The purpose of the experiments described in this article was to study the value of this hypothesis by ascertaining whether leucocytes contain and secrete growth-promoting substances, and whether tissues and exudates in which they accumulate acquire the power of activating cell proliferation.

¹ Carrel, A., *J. Exp. Med.*, 1913, xviii, 287.

² Carrel, A., *J. Exp. Med.*, 1913, xvii, 14. ³ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317.

I.

Growth-Promoting Action of Leucocytic Extracts and Secretions.

Leucocytes were obtained from the blood of chickens 1 or 2 years old. After centrifugation, the leucocytes were removed from the surface of the red blood corpuscles, washed several times in Ringer solution, and placed in a small amount of distilled water. After 1 or more days in the ice box, the cell suspension was shaken with once

TABLE I.

Action of an Extract of Leucocytes on the Rate of Multiplication of Fibroblasts.

Experiment No.	Extract No.	Nature of tissue cultivated.	Culture No.	Width of ring of new tissue.		
				Leucocyte extract.	Embryo juice.	Ringer solution.
1	247	Embryonic heart.	247	4.0		2.0
2	247	" "	247	4.0		2.0
3	247	" "	247	5.5	6.0	
4	247	" "	247	5.0	6.0	
5	275	" "	276	5.0		2.5
6	275	" "	276	6.0		2.0
7	275	" "	288	3.5		2.0
8	275	" "	288	3.0		2.5
9	470	" "	490	4.0		2.5
10	470	" "	477	3.0		1.7
11	18488	" "	571	2.5	3.0	
12	18488	1685th passage fibroblasts.	571	4.0	3.0	
13	18488	1685th " "	575	3.0	3.0	
14	18488	1687th " "	587	5.0	6.0	
15	18488	First subculture from Experiment 14.	587	5.0	5.0	

or twice its volume of Ringer solution and centrifuged. The extracts were, therefore, more diluted than the embryonic juice generally used in tissue cultures. Their activity was tested against fragments of chick embryo heart and pure cultures of chicken fibroblasts. The medium was composed of one or two volumes of plasma and one volume of leucocytic extract. In the controls, the leucocytic extract was replaced by Ringer solution or by embryonic juice. The rate of growth was ascertained by measurements of the width of the ring of new tissue which had grown in 48 hours. Fourteen experiments were performed (Table I). Although the extracts were diluted, the

width of the growth was generally 100 per cent greater in the experiments than in the controls containing Ringer solution, and about the same as in embryonic tissue extract. It is evident that dead leucocytes set free substances which promote cell multiplication as do embryonic juices.

An investigation was then made as to whether living leucocytes may secrete growth-promoting substances such as are extracted from them after death. Long ago, growth-activating substances were found to be secreted by embryonic tissues *in vitro*.^{1,2} Pulp of embryonic tissue was cultivated in plasma and Ringer solution. The experiments were incubated, while the controls were kept in cold storage. After 1 or 2 days, the serum was extracted from the coagulum and its activity tested against fibroblasts. The activating power of the fluid extracted from the cultures preserved in cold storage was lower than that of the cultures kept in the incubator. It appeared that the tissues growing actively for a short time had modified their medium by secreting in it growth-activating substances. Similar experiments were made with leucocytes. A medium composed of practically equal parts of chicken plasma and hypotonic Tyrode solution was spread on the surface of the cover of Gabritschewski dishes. About thirty fragments of a film of chicken leucocytes were placed in the medium. The control contained no leucocytes. Both dishes were incubated for 48 hours, and large colonies of leucocytes spread into the clot. Then the serum was extracted from the coagulum and tested against a pure strain of fibroblasts. The fluid from the medium which had contained leucocytes was markedly more active. It was evident that, under the conditions of the experiments, leucocytes secreted a substance which activated the rate of growth of fibroblasts.

II.

Growth-Promoting Action of Inflamed Connective Tissue Extracts and Peritoneal Exudates.

If leucocytes be capable of setting free growth-activating substances *in vivo* as well as *in vitro*, tissues and exudates where they accumulate must acquire the power of accelerating cell multiplication. Therefore, an attempt was made to ascertain whether inflamed connective tissue contains growth-promoting substances. It is known that extracts of connective tissue stimulate cell proliferation only slightly.¹

However, control experiments were repeated. Subcutaneous connective tissue taken from a chicken was extracted with Ringer solution. The growth in the cultures containing the extract was about 25 per cent larger than in the controls containing only Ringer solution. In order that leucocytes could accumulate in the connective tissue, a focus of aseptic inflammation was produced by injecting a solution of dilute hydrochloric acid into fragments of sponge placed under the skin of chickens.³ The connective tissue around the foreign bodies became markedly thickened, although no abscess developed. Fragments of inflamed connective tissue were then removed and cultivated in plasma in order to ascertain whether they contained living leucocytes. They were soon surrounded by a large ring of ameboid cells and after 24 hours the cultures looked almost like those of Rous sarcoma. The inflamed connective tissue was sliced into a pulp, extracted with a small amount of Ringer solution, and the extract tested against fibroblasts and embryonic heart. The medium was composed of one or two volumes of plasma and one volume of extract. The controls contained Ringer solution or embryonic tissue extract. Although the inflamed tissue extracts were dilute, the width of the new tissue was more than doubled by their presence (Table II). There was no doubt that inflamed connective tissue contained substances capable of increasing the rate of multiplication of fibroblasts.

Similar experiments were repeated with peritoneal exudates. Injections of staphylococcus suspension, dog red blood corpuscles, turpentine, or bouillon into the peritoneum of chickens did not determine the production of an exudate. However, pus obtained from a turpentine abscess in a dog was injected into the peritoneum of two chickens, and an opaque fluid, orange-yellow in color, was aspirated from the abdominal cavity 2 days later. This fluid contained a large number of white blood corpuscles. After centrifugation, it was tested against embryonic heart tissue and fibroblasts. In twelve experiments, the width of new tissue was 100 per cent larger than that obtained in Ringer solution and about equal to that in embryonic juice (Table III). It was then certain that a peritoneal exudate containing many leucocytes had acquired the power of stimulating cell multiplication.

³All operations were performed under ether anesthesia.

TABLE II.

Action of an Extract of Inflamed Connective Tissue on the Rate of Multiplication of Fibroblasts.

Experiment No.	Extract No.	Nature of tissue cultivated.	Culture No.	Width of ring of new tissue.		
				Inflamed tissue extract.	Embryo juice.	Ringer solution.
1	213	Embryonic heart.	258	1.0		0.5
2	213	" "	258	1.5		0.5
3	213	" "	258	1.5		0.5
4	213	" "	263	4.2		2.7
5	213	1650th passage fibroblasts.	261	7.0	5.0	
6	213	1650th " "	261	5.5	6.0	
7	213	Embryonic heart.	269	3.0		1.0
8	213	" "	269	3.0		1.5
9	213	" "	269	4.0		1.5
10	311	" "	322	3.0		2.0
11	328	" "	332	4.0		2.0
12	328	" "	332	4.0		2.0
13	339	" "	349	1.5		0.8
14	339	" "	350	3.0		0.8

TABLE III.

Action of Peritoneal Exudate on the Rate of Multiplication of Fibroblasts.

Experiment No.	Exudate No.	Nature of tissue cultivated.	Culture No.	Width of ring of new tissue.		
				Exudate.	Embryo juice.	Ringer solution.
1	844	1719th passage fibroblasts.	846	5.0	5.0	
2	844	1719th " "	846	3.0	4.0	
3	844	Embryonic heart.	852	2.0		1.0
4	844	" "	852	2.0		1.0
5	850	" "	858	2.0		0.8
6	850	" "	858	1.5		0.5
7	850	" "	858	3.5		0.5
8	850	" "	858	4.5		0.5
9	850	" "	864	2.5	2.0	
10	850	" "	864	1.75	2.0	
11	866	1727th passage fibroblasts.	852	2.0		1.0
12	866	1727th " "	852	2.0		1.0

III.

DISCUSSION AND SUMMARY.

Two main facts were brought to light by the preceding experiments: first, the presence of growth-activating substances in the leucocytes; second, the setting free of these substances in tissues and fluids where leucocytes accumulate. The existence of growth-promoting substances within the body of the leucocytes was to be expected. Leucocytes are embryonic cells and it is well known that embryonic tissues contain substances which stimulate cell proliferation. But the experiments gave a direct experimental proof of this fact. Then, during the whole life and even in extreme old age, there is a supply of growth-promoting substances within the organism which is potentially capable of restoring the activity of the resting cells. Embryonic tissue juice, as is known, can rejuvenate cells which have ceased to multiply *in vitro* and show evidences of degeneration.¹ If the growth-activating substances of leucocytes can be transferred *in vivo* to tissue cells, they may play a similar rôle.

Therefore, it was important to find out whether the growth-activating substances were set free either by the secretions of the living leucocytes or by diffusion from the body of the cells after they were injured or dead, and whether this phenomenon occurred actually *in vivo*. Indeed, the idea that leucocytes secrete substances necessary to normal physiological processes is by no means new. Long ago, Ranvier described the lymph cells as mobile unicellular glands,⁴ and Renaut thought that their function was to bring to fixed cells the necessary food material.⁵ This rôle of the leucocytes was considered by him as absolutely essential. According to his theory, differentiated cells could not live in the absence of the lymph cells, which supply them with the substances required for their activity. The presence of such substances in the leucocytes was shown by our experiments and the growth-activating power acquired by inflamed connective tissue demonstrated that the leucocytes could actually bring these substances to the fixed cells.

¹ Ranvier, cited by Renaut, J., *Traité d'histologie pratique*, Paris, 1889, i, pt. 1, 79.

⁵ Renaut, J., *Traité d'histologie pratique*, Paris, 1889, i, pt. 1, 79, 94.

Under certain conditions, this property of the white cells of the blood may cause the resumption of the activity of tissues which are in a resting state. In the adult organism, the tissues have ceased to grow and the blood plasma has acquired marked inhibiting properties. But growth-promoting substances are still stored in leucocytes, glands, and muscle tissue. The leucocytes could supply fibroblasts or epithelial cells with the necessary food material if they were present where cell proliferation is needed. The existence of mechanisms causing leucocytes to invade tissues in need of repair is certain. The initiation of healing seems to depend on the coming of the leucocytes to the wounded tissue.¹¹ When they are missing, as happens when the wound is protected from all external irritation, cicatrization is greatly delayed. On the contrary, when staphylococci, turpentine, and other irritants are applied at the surface of the wound, granulations appear after less than 48 hours.⁶ These irritants, although different in nature, have the common characteristic of determining an inflammation of the tissues and the migration of leucocytes from the vessels to the surface of the wound. Possibly the white cells bring the substances which adult tissues require in order to cicatrize or regenerate. They would have the function of storing away the growth-promoting substances characteristic of embryonic tissues, and bringing them to the regions of the organism where they are needed.

IV.

CONCLUSIONS.

1. Leucocyte extracts, like embryonic tissue juice, possess the power of increasing the rate of multiplication of fibroblasts *in vitro*.
2. Leucocytes secrete substances *in vitro* which also promote cell multiplication.
3. Peritoneal exudate or connective tissue invaded by leucocytes acquires the power of increasing the rate of multiplication of fibroblasts.
4. Leucocytes are capable of bringing growth-activating substances to tissue cells. They may have the important function of promoting cell multiplication in the parts of the organism where they accumulate under certain conditions.

⁶ Carrel, A., *J. Exp. Med.*, 1921, xxxiv, 425.

CULTURES OF ORGANIZED TISSUES.

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PLATES 41 AND 42.

(Received for publication, May 31, 1922.)

It was shown by Thomson¹ that if a whole organ or a complete chick embryo 24 hours old was explanted to a culture medium, it increased markedly in size and no outgrowth of cells took place. If part of an organ, for instance a toe of a chick embryo, was explanted, an uncontrolled proliferation of cells began from the injured parts. When the basement membranes are injured, the cells begin growing out into the medium, whereas an uninjured organ continues to grow as a complete structure controlled by the laws of the organism. Thomson calls this "somatic growth." The increase of the organ stopped entirely after it reached a certain size. Possibly the cessation of growth was due to lack of absorption of the nutriment.

The experiments reported herein are in a way similar to those of Thomson. We used fragments of intestines from chick embryos which were about to hatch. At this period of embryonic development, the intestinal epithelium is assumed to possess its normal function.

I.

Technique.

The experiments were carried out in the following way. Chick embryos about 21 days old were taken out of the shell aseptically. The abdominal cavity was exposed and a fragment of the small intestine was extirpated and placed in Ringer solution. In some cases, the intestine was opened with a pair of fine scissors and a little strip was cut off and placed in the culture medium, which consisted of equal volumes of chick plasma and tissue juice. In other experi-

¹ Thomson, D., *Proc. Roy. Soc. Med.*, 1913-14, vii, Marcus Beck Lab. Rep., 71.

ments, the fragment of intestine was turned inside out, leaving the epithelium outside and the serosa inside the lumen. It was washed to free it from the meconial mucous secretions and transferred to the medium. After 48 hours incubation at 39°C., the culture chamber was opened and the fragment was picked out with the point of a cataract knife, or was aspirated in a pipette. Then it was washed for a minute or so, and transferred to a new medium. The small fragments of intestine brought about an extensive liquefaction of the clot, so that after 48 hours incubation, they were usually found floating in a cup-like excavation, surrounded with fluid.

II.

Description of the Cultures.

In some of the cultures, an outgrowth of connective tissue or epithelium from part of the fragment could be seen; in others, there was no uncontrolled growth whatsoever. After a few passages, the proliferation usually stopped and the epithelium could be seen growing all around the fragment, which was completely covered by epithelial cells after 48 hours. There was then no further uncontrolled growth. At this stage, the fragment had become spherical and its surface was as shiny as fresh mucous membrane. Under the microscope, it often appeared as a semitransparent body with a slightly denser central portion. In the early stage of life *in vitro*, numerous intestinal villi were visible and the individual cells of the cylindrical epithelium could easily be distinguished. Active peristaltic movements were observed around the free edge of the cuticula of epithelial cells. A broad, semicircular pseudopod was often observed to protrude from one cell, followed by a similar activity in the neighboring cells, whereupon the first one relaxed. It was also possible to perceive migration of the ameboid cells between the epithelial cells.

In many experiments, the active contraction of the intestinal muscles could be seen *in vitro*, even after a month's cultivation. These contractions appeared as slow as the peristaltic movement *in vivo* and were brought about by a slight cooling of the preparation.

The size of the spherical bodies, or "organisms," varied according to their age. In general, they tended to grow smaller as time went on.

When the tissue was transferred to a fresh medium, the body contracted markedly, due to cooling and irritation during the manipulation, but after incubation for an hour or two in the new medium, it relaxed and consequently increased in size. Shortly thereafter, liquefaction took place around the epithelium, which secreted a mucous fluid. In a few cases, the actual secretion could be seen to exude from the epithelial cells. At the same time, several ameboid cells could be seen wandering through the stomata of the epithelial coat. They usually deteriorated rapidly in the mucus which surrounded the fragment. In later states, the intestinal body became pellucid, and the coat of regular cubic epithelium could be studied thoroughly. Often it was possible to observe small appendicular cystic formations which appeared on the surface of the body within a few hours (Fig. 1).

III.

Histological Examination.

After a month's cultivation, the tissues were fixed in 2 per cent formol-Ringer solution, and sectioned in series. They were found in an excellent state of preservation. The cylindrical epithelium had grown all around the fragment (Fig. 2). The villi had disappeared and the surface of the body was very even and smooth. The epithelium in the deep Lieberkühn glands was well preserved in many cases, but did not seem to have any communication with the outside. The connective tissue cells had formed a stroma with numerous fibrillæ. There were no evidences of uncontrolled growth, as the epithelial cells did not go beyond their natural border-line, and there was no intermixing of the different cells.

It was supposed that bodies such as this would be able to live in a fluid medium. Therefore, fragments of intestines were cultivated in the usual way in chick plasma and tissue juice until they were entirely coated with epithelium. Then they were placed on the bottom of hollow slides and 3 or 4 drops of tissue juice were added. At the end of about a month, they were fixed, sectioned, and stained. There was a marked difference between those cultivated in the fluid medium and those in the solid medium. In the fluid medium, the villi were perfectly preserved, and very little stroma was left. In the

center of the "organism" only a faint shadow of the stroma remained. Just under the lining epithelium which appeared to be normal, were a large number of epithelial cells in a more or less ameboid state. Under this layer, an empty space could be seen which had probably been filled with liquid, and finally toward the center of the body, a very thin, loose stroma with single connective tissue cells.

The cylindrical epithelium was very well preserved. Its free surface formed a continuous and solid cuticula. This contact between the epithelial cells was only perfect in their peripheral part. Between the external and the basal parts of the cells, free spaces could be seen, as if they had been formed under the pressure of a liquid within the body. The epithelial border of one which had been kept in a fluid medium for a long time is shown in Fig. 3.

IV.

DISCUSSION AND SUMMARY.

An artificial organism, if one may so term it, composed of a complex of tissues, was cultivated for a long period of time. Small fragments of intestine from chick embryos 20 to 21 days old were placed in a suitable medium. The epithelium proliferated and completely covered the fragment of intestine after 4 to 6 days. A small body was thus formed, round or oblong in shape, surrounded by cylindrical epithelium and containing epithelial, connective, and muscle tissues, endothelium, and ameboid cells. After a month's cultivation *in vitro*, no necrosis had occurred. Therefore, it may be assumed that, through the intestinal epithelium, the medium supplied the intestinal tissue with sufficient nourishment. No uncontrolled proliferation took place after the epithelium had surrounded the entire fragment.

The cultivation of complex tissues will facilitate the study of the interactions of the different cells under various conditions. In some experiments, pure cultures of epithelial cells were grafted into such an "organism" without difficulty. The growth of malignant cells could be studied in the same way. When the "organism" was placed in a fluid medium, the epithelium remained normal but the stroma disappeared. It seems that plasma played an important rôle in the maintenance of the tissues in their normal condition.

V.

CONCLUSIONS.

1. Fragments of small intestine from a 21 day old chick embryo, cultivated in plasma and tissue juice, became completely surrounded with cylindrical epithelium.

2. After a month's cultivation in plasma and tissue juice, the tissues composing the mass were normal. It would seem that the necessary food material was absorbed by the epithelium from the culture medium.

3. When these masses were cultivated in embryo juice without plasma, the intestinal villi remained normal, while the stroma, connective tissue cells, and their fibrillæ progressively disappeared, leaving an epithelial cyst.

EXPLANATION OF PLATES.

PLATE 41.

FIG. 1. Experiment 2107-24. Section of an intestinal "organism" cultivated in embryonic juice without plasma. The epithelium is normal. The stroma is very loose and the cells are sparsely represented. A cystic formation is seen at *A*. $\times 150$.

FIG. 2. Experiment 1085-6. Section of an intestinal "organism" cultivated in plasma and embryonic juice for a month. $\times 200$.

PLATE 42.

FIG. 3. Experiment 2107-22. Section of an intestinal "organism" cultivated in embryonic tissue juice for a month. The loose stroma and the cubic epithelium with the dilatated spaces between the individual cells may be seen. $\times 272$.

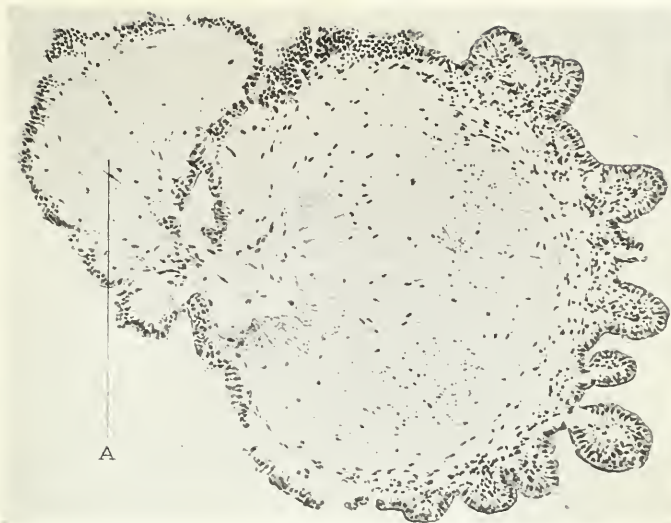


FIG. 1.



FIG. 2.

(Fischer: Cultures of organized tissues.)



FIG. 3.

(Fischer: Cultures of organized tissues.)

ACTION OF SHAKEN SERUM ON HOMOLOGOUS FIBROBLASTS.

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The activity of homologous fibroblasts decreases after the serum composing their medium has been heated at 56° and 70°C., while under the same cultural conditions, the rate of migration of heterologous fibroblasts increases.¹ The better growth of heterologous tissue in heated serum is probably due to the destruction of alexin. But the cause of the decreased activity of homologous tissue is still unknown. It may be attributed to the disappearance of alexin or of some other factor which should have, in common with alexin, the property of being thermolabile. Therefore, an attempt was made to ascertain whether factors other than heat might bring about simultaneous changes in the inhibiting action of serum on homologous fibroblasts, and in its alexinic activity. The purpose of the experiments described in this article was to study the effect of serum inactivated by shaking, on the growth of fibroblasts.

I.

Action of Shaken Serum on Foreign Erythrocytes and on Homologous Fibroblasts.

Serum was obtained from plasma of chickens about 1 year old, sealed in Pyrex tubes, and shaken at low speed for periods varying from 1 to 8 hours. It became more or less opalescent and, after centrifugation, a slight precipitate settled at the bottom of the tube. The volume of the precipitate varied according to individual differences in the animals. It was never very large, and the variations of the refractive index of the serum before and after shaking were

¹ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 647.

always slight. There was practically no difference in the H ion concentration of the fluid. The lytic action of serum on foreign cells was ascertained with sheep corpuscles. Fibroblasts obtained from

TABLE I.

Action of Shaken Chicken Serum on Homologous Fibroblasts and Sheep Erythrocytes.

Experiment No.	Serum No.	Time shaken.	Culture No.	Rate of growth of fibroblasts.		Hemolysis of sheep corpuscles.		
				Normal serum.	Shaken serum.	Concentration of serum.	Amount of hemolysis.	
							Normal serum.	Shaken serum.
		<i>hrs.</i>				<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	1406	1	1445	2.00	1.50	20	10	5
		1	1445	2.00	1.00	50	50	18
		1	1445	4.00	2.00			
		1	1445	3.00	1.50	70	90	35
2	1429	7	24967	3.48	2.97			
		7	24967	2.40	1.67			
		7	24967	3.59	2.50			
		7	25071	2.80	2.70			
		7	25071	3.54	2.89			
		7	25071	3.82	1.69			
3	1737	4	1741	4.76	1.39			
		4	1741	3.47	0.83			
		4	28374	3.36	2.62			
		4	28374	3.28	2.11			
		4	28374	3.21	2.72			
		4	28374	2.50	1.76			
4	1737	8	1750	1.60	0.01	10	45	0
		8	1750	2.26	0.01	20	100	0
5	1746	4	1751	3.19	1.82	20	80	70
		4	1751	3.90	1.01			
		4	1751	5.50	5.30	30	100	100
6	1758	4	1761	3.00	2.00			
		4	1761	2.00	1.50			
7	1758	7	1798	5.41	4.50	10	10	5
		7	1798	3.59	1.00	20	40	15
		7	1798	0	0	40	100	35

pure cultures of a 10 year old strain were used for measuring the effect of chicken serum on homologous cells. The medium consisted of one volume of normal plasma, two volumes of shaken serum, and one volume of shaken serum containing 1:20 embryo juice. In the controls, the shaken serum was replaced by normal serum. The rate of growth was measured according to a technique previously described.²

Five sera, shaken from 1 to 8 hours, were examined (Table I). They were partly inactivated as shown by the hemolytic tests. In one case, inactivation was complete. The inhibiting action of shaken serum on fibroblasts was always found to be at least 30 per cent greater than that of unshaken serum. In the experiment in which serum was completely inactivated, the differences in the action of both shaken and unshaken sera were still more marked (Table I, Experiment 4). It was evident that partial or complete inactivation by shaking had a marked restraining effect on the activity of homologous fibroblasts.

II.

Action of Shaken Serum on Foreign Erythrocytes and on Foreign Fibroblasts.

Fresh dog serum, shaken in the same manner as chicken serum, became slightly opaque and partly inactivated (Table II). Its in-

TABLE II.

Action of Shaken Dog Serum on Chicken Fibroblasts and Sheep Erythrocytes.

Experiment No.	Serum No.	Time shaken.	Culture No.	Rate of growth of fibroblasts.		Hemolysis of sheep corpuscles.		
				Normal serum.	Shaken serum.	Concentration of serum.	Amount of hemolysis.	
							Normal serum.	Shaken serum.
		<i>hrs.</i>				<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	1396	3	1402	3.5	4.5	10	100	25
		3	1402	2.5	4.0	20	85	7
2	1300	4	1423	2.0	8.0			
		4	1423	3.0	7.0			
3	1428	7	1434	2.0	3.5			
		7	1434	2.0	3.0			

² Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

hibiting action was tested upon chicken fibroblasts taken from stock cultures of a 10 year old strain, and found very much decreased. The average width of the ring of new tissue was about twice as large in shaken as in unshaken serum. It appeared that dog serum partly inactivated by shaking became a much better medium for chicken fibroblasts.

III.

DISCUSSION AND CONCLUSIONS.

The preceding experiments showed that partial or complete inactivation of serum by shaking brought about a marked decrease in the activity of homologous fibroblasts. Generally, serum was not completely inactivated by shaking. In only one instance was the hemolytic effect of chicken serum on sheep corpuscles entirely lost after the serum had been shaken for 8 hours. In all other cases, the normal hemolytic power of chicken and dog sera for sheep erythrocytes was merely decreased. The effect of shaking varied according to certain conditions of the serum. It may be compared to the influence of heat, which differs widely according to individual sera, even when they are obtained from animals which are apparently in identical condition. Shaken serum always inhibited the activity of homologous fibroblasts more than normal serum. When chicken serum was completely inactivated by shaking, its restraining action on chicken fibroblasts became also more marked. On the contrary, dog serum partly inactivated by shaking was much less toxic for chicken fibroblasts than normal serum. Thus, shaking brought about a change in the condition of serum, against which homologous and heterologous fibroblasts reacted in an opposite manner. At the same time, the normal lytic action of serum on foreign erythrocytes decreased.

This last phenomenon is caused, as is well known, by the partial or complete destruction of alexin. The decrease of the restraining effect of shaken serum on foreign fibroblasts may be attributed to the same cause.¹¹ The increase of the inhibiting action of shaken serum on homologous fibroblasts is due possibly to the disappearance of a substance favoring the activity of homologous cells. A similar hypothesis was advanced for explaining the increase of the growth-

inhibiting action of serum under the influence of senescence³ and of heat.¹⁴ The restraining power of adult serum on cell multiplication may be due to the antagonistic action of growth-promoting and growth-inhibiting substances, the growth-promoting substance being as unstable as alexin and certain tissue juices which have the property of increasing the rate of cell proliferation. ¹⁵Alexin and growth-activating substances contained in embryonic and gland tissue juices have in common the property of being destroyed by heat and by shaking. Leucocytes added to serum under certain conditions increase its hemolytic power on foreign erythrocytes and decrease its inhibiting action on homologous fibroblasts.¹¹ Variations in the alexinic activity of serum under other influences are followed also by a change in its action on homologous cells. Long ago, Gengou⁴ found that serum from plasma is less bactericidal than serum from blood. We observed that the lytic effect on sheep erythrocytes of dog and chicken serum from blood was sometimes more marked than that of serum from plasma, but there was often no difference in the action of both sera. At the same time, the growth of homologous fibroblasts was always more extensive in the serum from blood. The opposite effect was observed when heterologous fibroblasts were used. The growth-inhibiting power of serum from blood was still less marked on leucocytes than on fibroblasts. But embryonic juice, which greatly enhances the rate of multiplication of homologous cells, did not increase the lytic action of serum on foreign erythrocytes.

It may be concluded that, under the conditions of the experiments:

1. Chicken serum partly or completely inactivated by shaking becomes more inhibiting for chicken fibroblasts.
2. Dog serum partly inactivated by shaking becomes less inhibiting for chicken fibroblasts.

³ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.

⁴ Gengou, O., *Ann. Inst. Pasteur*, 1901, xv, 232.

THE HYDROGEN ION CONCENTRATION OF JOINT EXUDATES IN RHEUMATIC FEVER AND OTHER FORMS OF ARTHRITIS.

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(Received for publication, May 19, 1922.)

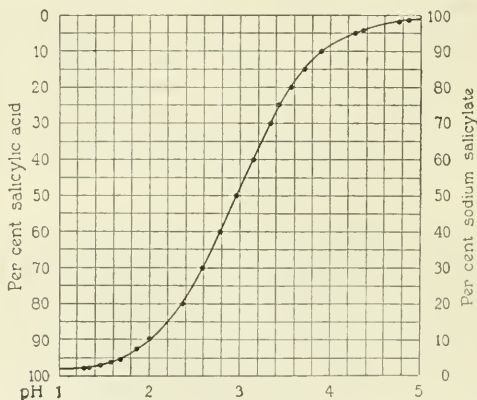
In connection with a study of acute rheumatic fever we have determined the hydrogen ion concentration of exudates aspirated from the inflamed joints of patients ill with this disease and also of exudates of patients with certain other forms of arthritis. This was done (1) to compare the reactions of the exudates in these arthritic diseases, and (2) to determine whether an acidity occurs in the inflamed joints in acute rheumatic fever sufficient to permit the liberation of free salicylic acid following salicylate therapy.

One explanation of the action of salicylates in patients with rheumatic fever has been based upon the hypothesis that free salicylic acid is liberated in the inflamed joint. If the inflammation in the joint results from the local irritation of bacteria, a bactericidal action of free salicylic acid might explain the improvement that ordinarily follows the administration of this drug. This theory originated with Binz (1), who realized that free salicylic acid could not exist in normal blood and tissues, and also that the salts of salicylates are not bactericidal in weak solutions. In subjecting sodium salicylate solutions to high CO₂ tensions *in vitro*, he found that sufficient acid was liberated to be bactericidal. The reason for the liberation of free salicylic acid in Binz' experiments is obvious: the increased CO₂ tension caused sufficient acidity to allow dissociation of the acid. He thought that a similar increased CO₂ tension occurred in the inflamed tissues in acute rheumatic fever because Ewald (2) had previously shown that such an increase could occur in certain types of inflammatory exudates. Although Binz' theory seemed unsatisfactory, it has never been conclusively disproven and is quoted in most pharmacologies.

Hanzlik and his collaborators (3), in an extensive investigation of the pharmacology of salicylates, examined joint fluids directly by the ferric alum test for the presence of free salicylic acid; the patients with rheumatic fever received full therapeutic doses of the drug. These authors found no free salicylic acid present. A criticism of this work was made by the authors themselves who state that: "Objection might be raised to the method used because of the possibility that

some CO_2 is lost when the fluid is exposed to the atmosphere, but this is to a considerable extent prevented by the presence of 'buffer' or protective substances."

Reaction at Which Free Salicylic Acid Occurs.—Hanzlik (4) also found that salicylic acid was released from mixtures of sodium salicylate and buffer salts only when the acidity was greater than pH 6.7. The iron test for salicylic acid was found to be faintly positive at a pH of 6.7 and progressively stronger with an increase in acidity from pH 6.5 to 1.0; on the alkaline side of pH 6.7 no salicylic acid was liberated. Mixtures containing 25 per cent serum



TEXT-FIG. 1. Salicylic acid-sodium salicylate curve at 40°C.

or plasma did not show any free salicylic acid between pH 7.4 and 5.9; tests were not carried out at a higher acidity. He states that: "It is conceivable that very low degrees of acidity, *i.e.*, in the neighborhood of pH = 6.8 or 6.9, might occur in closed cavities with sluggish circulation as in the articulations," but "fluids of joints and similarly enclosed regions would need to be more highly acid and freer from protein and other constituents than is probably the case in order to contain free salicylic acid and explain the therapeutic relief from salicylate medication according to the antiseptic theory."

The dissociation curve for mixtures of salicylic acid and sodium salicylate is given in Text-fig. 1. It is evident that at reactions more

alkaline than pH 5.0 the free acid constitutes less than 1 per cent or the total salicylate. Since it has been estimated that following full therapeutic doses of salicylate the concentration in the blood or joint fluid is about 0.02 per cent (3), the amount of free acid present at a pH of 6.0 would be less than 0.0002 per cent; *i.e.*, an amount that could exert no bactericidal action. This conclusion is in agreement with Hanzlik's experimental findings.

EXPERIMENTAL.

The reactions of twenty-six joint exudates have been determined. The majority of these were from patients ill with acute rheumatic fever; a number were from patients having arthritis of unknown origin (chronic arthritis, intermittent hydrarthrosis); two were from patients with definite bacterial arthritis; and one, a simple effusion, was from a patient with myocardial insufficiency and generalized edema.

In each instance the joint was aspirated with a tightly fitting Luer syringe containing a small amount of sterile paraffin oil to prevent the admission of any air bubbles. During the entire determination the fluid was prevented from coming in contact with the air.

Owing to the fact that a considerable quantity of fluid was necessary for electrometric pH determinations and that in each instance several additional cubic centimeters were needed for culture, colorimetric determinations were made in the majority of instances. When large quantities of fluid were available, both colorimetric and electrometric determinations were made, in order to obtain the factor necessary to convert colorimetric readings at room temperature to electrometric at body temperature (38°C.).

*Electrometric Measurements.*¹—The electrometric determinations were made at 38°C. in the Clark cell, in a hydrogen atmosphere containing CO₂ at the tension existing in the joint fluid. The determinations were corrected for partial pressures of CO₂ and H₂O to one atmosphere of dry H₂. The solution used in the standardization of the hydrogen electrode was 0.1 N HCl whose pH at 38° was assumed to be 1.09. This brings the pH determinations to the basis of Sören-

¹ A number of these determinations were made by Dr. A. B. Hastings.

sen's standards. A detailed description of the technique employed has been recently published by Cullen (5).

Colorimetric Measurements.—The method was that described by Cullen (5) for the colorimetric determination of the hydrogen ion concentration of blood. 1 cc. of the aspirated joint fluid (usually within 4 minutes of withdrawal and before coagulation occurred) was added to a standard tube containing 20 cc. of 0.9 per cent sodium chloride solution and 7 drops of indicator; the addition was made under paraffin oil to prevent the escape of any CO_2 . 1 cc. of the same joint fluid was added to another tube containing 20 cc. of saline solution without any indicator to serve as a turbidity control in the comparator. The pH determination was then made by placing the tube in a comparator block and comparing it with standard color tubes. This was done at room temperature. Phenol red was used as indicator for determinations above pH 6.8 and brom-cresol purple for those below pH 6.8. All glassware, mineral oil, and salt solution were previously tested for neutrality. As standards, Sørensen's phosphate standards were prepared in steps of pH 0.2 from pH 5.6 to 6.8 and in steps of pH 0.05 from pH of 6.8 to 8.0. To those above pH 6.8, 5 drops of 0.03 per cent phenol red solution were added to each 15 cc. of standard; to those below pH 6.8 a similar quantity of brom-cresol purple was added.

The colorimetric determination at room temperature was corrected to 38°C. by the following formula:

$$\text{pH}_{38} = \text{colorimetric pH} + 0.01 (t - 20^\circ) \text{ pH} - 0.21 \text{ pH}$$

in which t = room temperature and 0.21 pH represents the empirical correction for temperature ($38^\circ - 20^\circ$), dilution, and protein errors. This correction represents the average of the differences between electrometric determinations at 38°C. undiluted, and the colorimetric determinations, diluted, at 20°C.; it corresponds closely with the correction similarly found for correcting colorimetric determinations of human blood. The room temperature in these experiments varied within $\pm 3^\circ\text{C.}$ from 20°C. and a temperature change of 0.01 pH was made for each degree of variation.

Physical Characteristics of the Exudates Examined.—The fluids aspirated from the inflamed joints of patients ill with acute rheumatic

fever were never frankly purulent. They were viscous, slightly to distinctly turbid, and usually of a pale yellowish green color. Sometimes when the exudate was aspirated from a joint during the early stage of the arthritis, the greenish yellow shade was absent and the fluid had a grayish turbidity; this effect was probably due to a higher leucocyte content. The exudates contained considerable fibrin and formed soft clots on standing. Bacteriologically, they were sterile by ordinary culture methods. The fluids from the patients with arthritis of undetermined origin were indistinguishable from some of these exudates. The joint fluid from the patient with myocardial insufficiency was less turbid, did not clot so quickly, and had a lower leucocyte count than most of the rheumatic fever exudates. The exudates of the two patients with bacterial arthritis were easily distinguished as coming from infected joints. They were purulent fluids and contained bacteria.

Results.

Hydrogen Ion Concentration.—The results are shown in Table I. With the exception of the joint exudates of the two patients with bacterial arthritis, the reactions were all slightly alkaline and approximated the normal reaction of blood. The hydrogen ion determinations of sixteen fluids from patients with acute rheumatic fever varied between pH 7.27 and 7.42. Seven fluids were examined from three patients with arthritis of undetermined origin; one of these was diagnosed as intermittent hydrarthrosis, one as chronic arthritis, and the third was probably arthritis accompanying serum disease. Their reactions were approximately the same as those in acute rheumatic fever, varying between pH 7.33 and 7.47. The joint fluids of the two patients with bacterial arthritis were both definitely acid; the exudate from the knee joint infected with *Staphylococcus aureus* was pH 6.69; from the one with *Streptococcus hemolyticus*, pH 6.19. The electro-metric determination of this latter fluid cannot be considered accurate as the fluid was kept over night in the ice box and heated to 56°C. for 1 hour to kill the organisms; the result, however, is sufficiently accurate to show the fluid to be definitely acid. The simple effusion from the patient with myocardial insufficiency accompanied by general edema was pH 7.34.

TABLE I.

Hydrogen Ion Concentration of Joint Exudates.

Case No.	Patient's temperature at time of aspiration.	Joint.	Length of time joint aspirated was involved.	Degree of inflammation.*	Bacteriological examination.	Determinations.			
						Colorimetric at 20° C.	Electrometric at 38° C.	Difference between colorimetric at 20° C. and electrometric at 38° C.	Colorimetric calculated at 38° C.
Acute rheumatic fever.									
	°F.		days			pH	pH	pH	pH
4444	104.5	Right knee.	3	+++	No growth.	7.65	7.39	0.26	7.39
4350	102	Left wrist.	5	+	" "	7.53‡			7.32
4453	102	Right knee.	1	+++	" "	7.58	7.34	0.24	7.34
	99	Left "	7	+	" "	7.58			7.37
4367	100.5	Right "	10	+	" "	7.63‡			7.42
	104	Left "	2	++	" "	7.56‡			7.35
	104	Right "	2	++	" "	7.57‡			7.36
4417	103	" "	8	+	" "	7.54‡			7.33
	103	Left "	3	++	" "	7.54‡			7.33
	102.5	" "	5	+	" "	7.55‡			7.34
	102.5	Right "	12	+	" "	7.57‡			7.36
4473	103	Left "	1	+++	" "	7.49	7.27	0.22	7.27
	103	" "	2	++	" "	7.60			7.39
4485	103.8	Right "	1	+++	" "	7.54			7.33
	103.8	Left "	3	++	" "	7.56			7.35
4493	103	" "	4	+++	" "	7.51	7.34	0.17	7.34

* + indicates swelling only; ++, moderate inflammation; +++, marked inflammation.

† Average correction of pH 0.21 used except where actual difference is given.

‡ Room temperature not recorded. Variation not more than $\pm 3^\circ\text{C}.$ from $20^\circ\text{C}.$

TABLE I—*Concluded.*

Case No.	Patient's temperature at time of aspiration.	Joint.	Length of time joint aspirated was involved.	Degree of inflammation.*	Bacteriological examination.	Determinations.			
						Colorimetric at 20° C.	Electrometric at 38° C.	Difference between colorimetric at 20° C. and electrometric at 38° C.	Colorimetric calculated at 38° C.†
Arthritis of undetermined origin (includes chronic arthritis and intermittent hydrarthrosis).									
	°F.		days			pH	pH	pH	pH
4451	101	Right knee.	2	+++	No growth.	7.54			7.33
	102	Left "	5	+++	" "	7.65			7.44
4428	99	Right "	?	+	" "	7.61	7.43	0.18	7.43
	100	" "	2	+	" "	7.67	7.42	0.25	7.42
	99	" "	2	+	" "	7.62	7.42	0.20	7.42
	98.5	" "	30 mos.	+	" "	7.68			7.47
4449	99	Left "	5	+	" "	7.60	7.42	0.18	7.42
Bacterial arthritis.									
4262	104	Right knee.	8	+++	<i>Staphylococcus aureus.</i>	6.90			6.69
4467	104.2	Left "	?	+++	<i>Streptococcus hamolyticus.</i>	6.40§ 6.10	5.89	0.21	6.19
Joint effusion, myocardial insufficiency.									
4458	102	Left knee.	?	+	No growth.	7.55			7.34

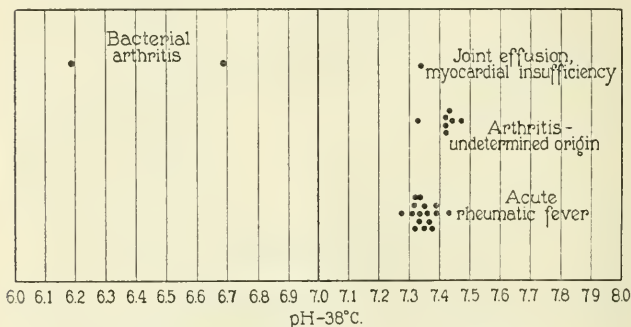
§ Within 4 minutes of aspiration.

|| Determined after standing in ice box over night and heated at 56°C. for 1 hour to kill bacteria.

DISCUSSION.

A comparison of the results is indicated in Text-fig. 2. It shows that the joint exudates on the basis of their reactions fall into two groups; the one having a slightly acid, the other a slightly alkaline reaction. The fluids containing bacteria fell into the first, the sterile fluids into the second. In general, these findings correspond with those of other investigators who have recently made hydrogen ion

determinations on various types of exudates. Shearer and Parsons (6) found the purulent spinal fluid of epidemic cerebrospinal meningitis to be about pH 6.9. Lord (7) found the pH of pneumonic lung exudates to be as low as 5.4. Shade and his coworkers (8) determined the reactions of many exudates electrometrically; pus from acute infections varied in pH between 5.96 and 6.57; pus from chronic inflammations such as tuberculosis between 6.58 and 7.00; serous exudates as in tuberculous pleurisy between 7.00 and 7.09; and non-inflammatory transudates between pH 7.17 and 7.24.



TEXT-FIG. 2. Hydrogen ion concentration of joint exudates.

It seems to us that only in the instance of purulent exudates would the liberation of free salicylic acid be at all possible. If acid is liberated in these exudates it is not sufficient to be bactericidal, as clinical observations have never shown any improvement in purulent arthritis following salicylate treatment. As Hanzlik has previously concluded from direct examinations that free salicylic acid could not be demonstrated in the joint exudates of patients with acute rheumatic fever, and our findings indicate that free acid could not possibly exist, it is evident that the local antiphlogistic effect of the drug is due to some other factor than free salicylic acid.

The determination of the reactions of these fluids has thrown some light upon the nature of the pathological process in the arthritis of

acute rheumatic fever. The results of various workers already mentioned show that when bacteria such as hemolytic streptococci, staphylococci, pneumococci, meningococci, or tubercle bacilli are present in an exudate in sufficient numbers to be detected, either by cultural or microscopic examination, the reaction of that exudate is always more acid than blood. We have evidence to show that the exudates in experimental arthritis of animals inoculated with green streptococci are also acid. From these results it would seem that if green streptococci were growing in the joint fluids of rheumatic fever patients, one should expect those fluids to be acid. But our examinations have shown that this is not the case. It is highly improbable, therefore, that one could cultivate streptococci from such exudates. This is in agreement with our bacteriological studies.

Another possibility must be considered: the etiologic agent—whatever it may be—may exist only in the capsule or contiguous tissues; and the exudate may be similar in nature to the serofibrinous fluid often found in pleurisy secondary to pneumonia. In many instances these pleuritic fluids are sterile and the patients recover without developing empyema. While there are not sufficient data concerning the reaction of such fluids, from the report of Shade we would expect them to be more alkaline than pH 7.0. Further observations should be made concerning the reaction of sterile fluids in serous cavities contiguous to foci of inflammation resulting from bacterial infection. When this information is available we shall be in a better position to evaluate properly the relation of our findings to the etiology and pathology of rheumatic fever.

The slight variation in the reactions of the exudates of acute rheumatic fever was not dependent upon the severity of the inflammation as evidenced clinically. The exudates from acutely inflamed joints were of about the same pH as those from joints in which the clinical signs of acuteness had disappeared and the only remaining evidence of involvement was the presence of fluid. The most acid exudate, having a pH 7.27, was found in a highly inflamed joint; the most alkaline fluid, having a pH 7.42, was found in a joint from which all acute signs of inflammation had disappeared; one highly inflamed joint, however, had a pH of 7.39 (Table II).

TABLE II.

Acute Rheumatic Fever.

Comparison of Exudates from Acutely Inflamed Joints with Those in Which Signs of Acute Inflammation Had Subsided.

Exudates from joints acutely inflamed.....	pH _{38°} =7.27—7.39
“ “ “ in which inflammation had subsided.....	pH _{38°} =7.31—7.42

SUMMARY AND CONCLUSIONS.

1. The hydrogen ion concentration of joint exudates aspirated from patients ill with acute rheumatic fever, arthritis of undetermined origin, and bacterial arthritis was determined. The hydrogen ion concentrations of the joint exudates from patients with acute rheumatic fever approximated the normal reaction of blood, varying from pH 7.27 to 7.42. Exudates from patients with arthritis of undetermined origin varied in pH from 7.33 to 7.47. The pH of a joint effusion occurring in a patient with myocardial insufficiency was 7.34. Bacteriologically, all of these fluids were sterile by ordinary means of cultivation. An exudate aspirated from a knee infected with *Staphylococcus aureus* had a pH of 6.69, while that from a patient having an arthritis due to *Streptococcus hæmolyticus* was also acid, having a pH of 6.19.

2. Since a definitely acid medium is necessary for the liberation of free salicylic acid and since all of the joint fluids from patients with acute rheumatic fever were slightly alkaline, no free salicylic acid could possibly exist in such joint fluids following the administration of salicylates.

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ON ALIMENTARY LEUCOCYTOSIS IN ITS RELATION TO THE "CRISE HÉMOCLASIQUE" OF WIDAL.

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During the last few years Widal and his coworkers have shown that under certain conditions a characteristic complex of changes occurs in the blood, a complex denoted by Widal as "*crise hémoclasique*," or "*crise colloïdoclasique*."

If a normal animal is injected intravenously with a certain dose of peptone the blood pressure will fall, the clotting time of the blood will be decreased, the number of leucocytes in the peripheral blood stream will decrease, and the refractive index of the serum will change. Widal (1) showed that in the normal dog after a protein meal higher cleavage products of protein, *i.e.* peptones or albumoses, are absorbed from the intestine, and the occurrence of these cleavage products in the portal blood stream can be demonstrated by physiological means. In normal animals these cleavage products cannot enter the general blood stream, since they are fixed in the liver ("*pouvoir protéopexique*" of the liver). If, however, the liver cells are damaged, they lose part of their fixing power, and albumoses or peptones will escape in the general blood stream and will produce changes in clotting time, in refractive index, in leucocyte count, and so forth, and thus a *crise hémoclasique* will ensue. Such a *crise hémoclasique* can also be produced in other ways; *viz.*, by injection of a specific protein into an individual sensitized to that protein (anaphylactic shock), by injections of certain colloids or of drugs, such as salvarsan, to predisposed individuals, etc. The main point of interest, with regard to the work published in the present paper, is the observation of Widal and his coworkers (2) that in cases of hypersensitiveness to certain foodstuffs in men the ingestion of these foodstuffs will produce a *crise colloïdoclasique* even in the absence of any demonstrable damage of

liver cells. The interrelation of symptoms of hypersensitiveness and of those of a *crise hémoclasique* is, according to Widal, so close that in doubtful cases the finding of a *crise hémoclasique* after ingestion of a certain foodstuff will give an indication as to the nature of the causative agent of the symptoms of hypersensitiveness (migraine, asthma, hay fever, etc.) and thus clear up the diagnosis. Since, according to Widal, the most prominent feature of the *crise hémoclasique* is the decrease in leucocyte count within 20 to 40 minutes after ingestion of the foodstuff or drug, in many cases the diagnosis of hypersensitiveness can be made from a series of leucocyte counts at intervals of 20 minutes before and after the ingestion of a certain foodstuff.

Two of the present authors have been interested in the study of the causes and treatment of bronchial asthma. They found that in many cases of bronchial asthma and hay fever there seems to be a relation between hypersensitiveness to tuberculin and hypersensitiveness to the known or unknown causative agent of the acute attacks of asthma, and were able to prove that in many of these cases a cure or a considerable relief of the asthmatic symptoms could be obtained by desensitizing the patients to tuberculin by means of repeated injections of small doses of this (3). In the course of this work, however, they obtained the impression that in some of the asthma patients who were resistant to the tuberculin therapy the cause of the attacks of asthma was to be looked for in the intestine, and later it appeared that in a number of other cases who were benefited by tuberculin treatment an intestinal factor also played an important part. In accordance with the current opinion the outbreak of attacks of asthma in these cases was ascribed to the ingestion of one definite protein (varying from case to case, but being specific for each patient), to which the patient in question was hypersensitive. It may be stated at once that this opinion proved to be incorrect. Observations and deductions on this point will be discussed in another paper. They only wish to emphasize here that they had at that time to face the following problems:

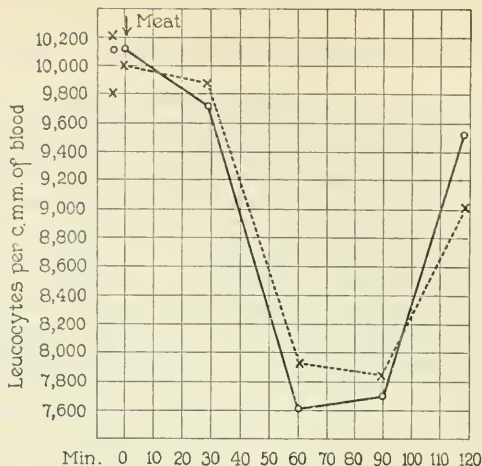
Taken for granted that certain symptoms of hypersensitiveness (attacks of asthma) in an individual are caused by the ingestion of a definite, but unknown, protein, how was this protein to be identified? Obviously the most exact way would be to feed the patient for a couple

of days on one protein only, changing after some days to another protein and going on in this way till the protein causing the attacks of asthma was found. This procedure, apart from offering considerable technical difficulties, would take a long time and, moreover, would suffer from the psychic factors which are of great influence in many cases of asthma.

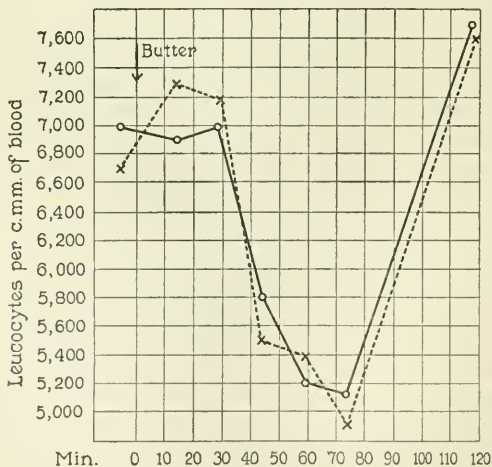
After having studied the work of Widal and his coworkers it seemed possible that the simple method indicated by him for the determination of the occurrence of a *crise colloïdoclasique*, i.e. counting of leucocytes in the peripheral blood stream, might be adopted for our cases. Consequently a study was undertaken of the influence of ingestion of various proteins on the leucocyte counts in a number of asthma patients. After the first investigations it proved to be necessary to include a study of other foodstuffs, viz. fat and carbohydrates, and to extend our investigations to the behavior of normal men. The result of this work which includes 150 observations on 50 individuals is given below.

In our first tests we followed exactly the instructions given by Widal. Leucocyte counts were made in the morning before any food had been taken, then the patient took the food to be tested, and during the next 2 hours counts were made at intervals of 20 minutes. At first three kinds of test meals were used; viz., 300 cc. of milk, or three eggs, or 150 gm. of meat. Since, however, clinical observations had shown that in some of our asthma patients attacks of asthma were produced also by the ingestion of non-protein food, the investigation was extended to butter (100 gm.) and rice (150 gm.).

About the technique employed only a few words need be said. Blood was taken from the finger-tip and counts were made in a Bürker counting chamber (so that we used about the same method as Widal). In all observations to be reported here double counts were made; that is to say, either one investigator took blood in two pipettes and used two counting chambers and afterwards plotted the results obtained in each counting chamber separately, or blood was taken in two pipettes by two investigators each of whom made counts in one chamber. We wish to emphasize that the curves obtained in this way run almost entirely parallel; the technical error is certainly not higher than 5 per cent (Text-figs. 1 and 2), whereas those changes



TEXT-FIG. 1. Leucocytic counts made at the same time by two different observers. The arrow indicates the ingestion of 150 gm. of meat.



TEXT-FIG. 2. Leucocytic counts made in one experiment by one observer working with two pipettes and two counting chambers. The arrow indicates the ingestion of 100 gm. of butter.

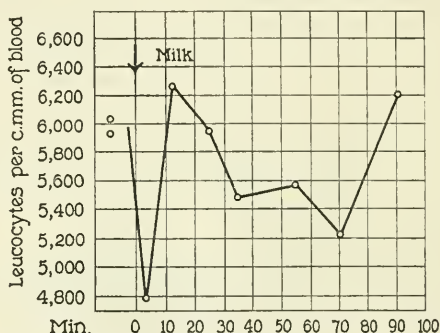
in leucocyte counts which are to be considered of clinical significance are about 20 per cent and higher, so that the method used, although not absolutely accurate, was sufficient for our purpose.

In nearly every case of asthma we found a *crise hémoclasique* after ingestion of some test meal but every case gave different results. Some reacted to milk only, others to meat, or butter, eggs, or rice only, still others reacted to butter and rice or to meat and eggs; in fact nearly every possible combination was found. Text-fig. 1 gives an instance of a definite fall in leucocytic counts after ingestion of meat, Text-fig. 2 after butter in another patient.

The fact that a fall in leucocytic count was found after ingestion of butter as well as after ingestion of protein showed that Widal's explanation as to the origin of the *crise* certainly could not hold good in our cases, since it is not very likely that ingestion of butter would cause after 20 minutes an increase in the albumose content of the portal blood stream. It is fair to state here that we are not the first to show that a fall in the number of leucocytes in the peripheral blood may occur after the ingestion of non-protein matter, as Widal has already found the same phenomenon after the ingestion of small amounts of glucose in diabetics (1).

Apart from this consideration, another observation was made which opened the question whether a leucopenia found in many of the cases in which the test meal consisted of protein could be explained on the same basis as in Widal's cases. Thus a considerable fall in the number of white blood cells (to more than 20 per cent below the original number) ensued within much less than 20 minutes after the ingestion of the food, so that it seemed hardly possible that in so short a time protein matter would be decomposed to albumoses and absorbed so as to produce a *crise*. This led us to decrease gradually the interval between the ingestion of the food and the first counting following it, so that in the end this interval was only 2 minutes. It was surprising to find that even in this last case a fall in the number of leucocytes occurred in some individuals. Text-fig. 3 gives an instance of such an incident. Before the ingestion of food the number of leucocytes was 6,000 per c.mm. The patient drank 200 cc. of milk, and 2 minutes afterwards blood was taken from a finger-tip; the number of white cells fell to 4,800; *i.e.*, a fall of about 20 per cent.

The end of this curve is equally interesting; it shows that 10 minutes after the ingestion of milk the number of leucocytes was about normal again; 10 minutes later, *i.e.* 20 minutes after the taking of milk, the curve shows a tendency to go down again, and gradually the number of leucocytes decreases and seems to reach a (second) minimum about 70 minutes after the drinking of the milk. Text-fig. 4 gives a similar curve after a test meal of rice. The original count of white cells was 9,000 in this case; 2 minutes after the eating of rice the leucocytes fell to 6,800, *i.e.* a drop of about 25 per cent; during the next three counts, made at intervals of 3 minutes, they increased to about 8,200,

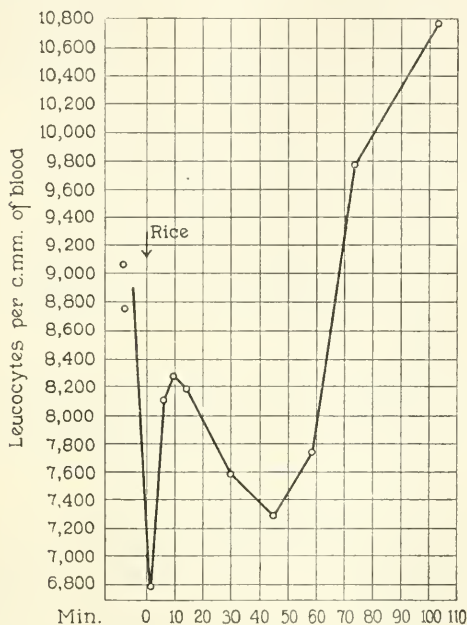


TEXT-FIG. 3. Decrease in the number of leucocytes within 2 minutes after the ingestion of milk. The arrow indicates the ingestion of 200 cc. of milk.

then followed a second drop, and subsequently a rise, far above the original number. This rise in leucocytes, which often occurs if counts are made during a sufficiently long time, is nothing else than the so called physiological leucocytosis after ingestion of food and will be left out of consideration, since the phenomena which are of interest here all occur before this physiological leucocytosis sets in.

The fact that a fall in number of white cells often occurs within 2 minutes after the ingestion of food excludes any possibility that *this* fall has a connection with absorption of cleavage products of the food administered; very likely it is caused by some reflex action from the alimentary canal, and we consider it highly probable that the phenom-

enon is only an expression of a change in the distribution of the white cells in the different regions of the blood stream. This opinion is strengthened by two observations; first, it was found that during this initial fall no characteristic change in the leucocyte formula



TEXT-FIG. 4. Decrease in the number of leucocytes within 2 minutes after the ingestion of rice. The arrow indicates the ingestion of 150 gm. of cooked rice.

occurs, and secondly, it was shown that the red cells show a decrease in number nearly parallel with that of the white cells.

The considerations mentioned made it probable that the fall in the number of leucocytes observed in the asthmatics was not to be considered as an expression of a pathological state in the individual

tested, but merely as a physiological occurrence. The correctness of this surmise was proved by finding exactly the same decrease in the number of leucocytes in normal individuals after a test meal of milk, meat, rice, and other foodstuffs. From this investigation on normal individuals it was found that in many cases a curve obtained by plotting the number of leucocytes of the peripheral blood counted at very short intervals after the ingestion of food will show the characteristics found in Text-fig. 3; *i.e.*, shortly after the test meal a sharp fall in white cells occurs. As this fall may last a few minutes or even a little longer, it is uncertain whether it will be detected if counts at intervals of 20 minutes are made. After the first fall comes a rise to about the original level, and subsequently there is a gradual fall followed by a final rise (physiological leucocytosis).

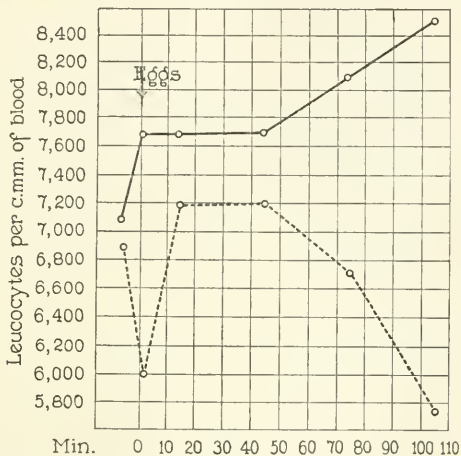
It must be stated, however, that although this type of curve frequently occurs, in other instances the curve may show a different shape. There may occur a sharp rise after some minutes, instead of a fall, or the second minimum may be absent and even replaced by a rise, and so forth.

The facts described above are in agreement with the findings of Dorlencourt and Banu (4) who studied digestive leucocytosis in normal infants. They found a decrease in leucocytes immediately after the ingestion of milk; this was followed by an increase and subsequently by a second decrease. Only after this period had passed, did the real digestive leucocytosis set in.

Adelsberger (5) and Schiff and Stransky (6) also found in normal infants and children a decrease in the number of leucocytes of short duration following shortly after the ingestion of milk and other proteins, fats, and carbohydrates.

After it was found that in many cases ingestion of food, either of protein or of non-protein nature, may cause an initial fall in the number of white cells, which may last for 5 to 20 minutes, and which may be followed first by a rise and afterwards by a second fall eventually followed by a second rise, and since it was also found that the time during which these falls and rises are demonstrable may vary from case to case, the question arose whether curves obtained by plotting the results of blood counts made at intervals of 20 or 30 minutes gave a true picture of the change in the number of leucocytes occurring in

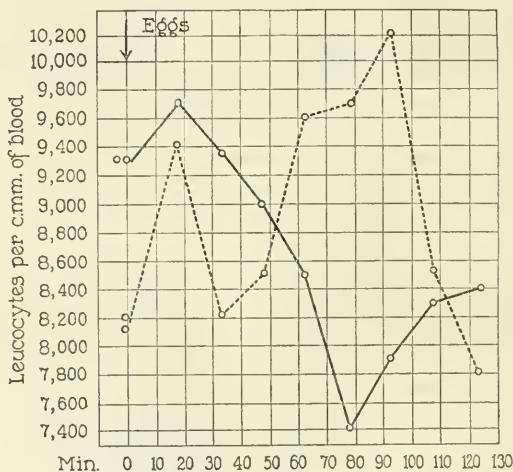
the blood. In fact it seemed very likely that results obtained in this way might be to a large degree dependent on mere chance, and the finding of a decrease of the number of white cells after the ingestion of some foodstuff could not be considered as an indication of the presence of a *crise hémoclasique* nor be an indication that this particular foodstuff was in any way related to pathological phenomena occurring in the body. To test this supposition control estimations



TEXT-FIG. 5. Leucocytic counts made by the same observer with the same food on the same patient but on 2 different days. The arrow indicates the ingestion of three eggs.

were carried out on a number of patients and normal individuals. In these cases blood counts were made at intervals of 20 minutes after the ingestion of eggs or other foodstuffs, and 2 or 3 days later the tests were repeated on the same individual, with the same foodstuffs. The curves obtained on 2 different days sometimes agreed with each other, but often also there was a considerable disagreement (Text-figs. 5 and 6). In Text-fig. 5 the first observation revealed an initial fall of leucocytes of about 13 per cent followed by a second fall of

about 20 per cent. In the second observation on the same person with the same food, only a rise in leucocytes was found. Text-fig. 6 shows a marked discrepancy between the findings on 2 different days. Attention should be called to the fact that these discrepancies cannot be ascribed to technical errors since two estimations on the same patient on the same day, even if the countings were made by different investigators, showed a nearly complete agreement (see Text-fig. 1).



TEXT-FIG. 6. The same experiments as in Text-fig. 5 but on another patient.

DISCUSSION.

Before entering into a discussion of the results we want to state that our work does not enable us to criticize Widal's conception of an interrelation of symptoms of hypersensitiveness, or anaphylactic shock, with the occurrence of a *crise hémoclasique* (*colloïdoclasique*). In all their former work and in the main part of their later work Widal and his coworkers founded their diagnosis of *crise hémoclasique* not on *one* symptom but on a complex of four or more symptoms. Later

Widal stated that since the occurrence of a fall in the number of white cells in the peripheral blood was the most prominent part of the *crise*, it was sufficient for the diagnosis of the same; we believe that our investigations have proved this last statement to be incorrect. We found a fall of white cells at various periods—from 2 to 50 minutes—after the ingestion of a meal in normal individuals and asthmatics. Moreover, a study of the blood pressure in some of these cases at short intervals showed an absence of a fall in pressure, so that there is no doubt that in our cases the fall of leucocytes in the peripheral blood was not an indication of the existence of a *crise colloïdologique*.

Soon after the appearance of Widal's papers on the *crise colloïdologique* a number of other workers started investigations as to the occurrence of *crises* in various conditions. Often the only criterion used for the diagnosis of a *crise* was the counting of the white blood cells. It is clear that as a consequence of our studies related above, those results can no longer be considered as conclusive. The fundamental observations of Widal and his coworkers, however, remain untouched by our work.

CONCLUSIONS.

Counts of the number of white blood cells at short intervals after the ingestion of a meal (meat, eggs, milk, rice, or butter) in normal individuals and in a number of asthmatics reveal the following facts.

As a rule, a sharp fall in the leucocytic curve occurs within 1 or 2 minutes after the meal; generally the curve rises within 10 to 20 minutes, but often a second fall follows 30 to 50 minutes after the meal. This may be followed by a slow rise in the curve (physiological leucocytosis). The first sharp fall is often accompanied by a similar decrease in red cells, the leucocytic formula is not changed, the blood pressure also remains unchanged, and this makes it probable that the leucopenia observed is only a manifestation of a change in distribution of the blood in different regions of the body.

Not infrequently the leucocyte curve after ingestion of food shows a form differing considerably from that described above. Counts of white cells made at intervals of 20 minutes in the same patient at different times but after ingestion of the same food show very differ-

ent leucocytic curves. Such counts do not give evidence of the existence of a *crise hémoclasique* and consequently cannot be used to identify the causative agent of cases of hypersensitiveness to foodstuffs or drugs. Whether such an identification can be obtained if instead of simply counting white cells the whole complex of symptoms originally described by Widal as characteristic for a *crise hémoclasique* is used, remains undetermined by our work.

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EXPERIMENTAL RICKETS IN RATS.

VII. THE PREVENTION OF RICKETS BY SUNLIGHT, BY THE RAYS OF THE MERCURY VAPOR LAMP, AND BY THE CARBON ARC LAMP.

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PLATE 43.

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It is now well established that infantile rickets can be prevented or cured by means of sunlight, or light from various artificial sources. Recently it has been shown by Hess, Unger, and Pappenheimer (1), and by Powers, Park, Shipley, McCollum, and Simmonds (2), that light is able to exert a similar favorable influence on the experimental rickets of rats. For the past year a large series of experiments, some of which have been reported in abstract as the work progressed, has been carried out in this laboratory with sunlight and rays of different kinds under a great variety of conditions. The investigation has included a study of the effect of variation of intensity, of transmission, or of reflection of light, of duration of exposure, of temperature, of the diet of the experimental animals, and the pigmentation of the skin, as well as other factors. A summary of the results of many of these experiments is incorporated in Tables I to VIII.

The Effect of Sunlight.

For all experiments young rats about 40 to 50 gm. in weight were used. They were kept in a darkened room at all times. After an interval of about 21 days they were radiographed for the appearance of rickets at the epiphyses of the knee joints, and were killed after a total period of 25 to 28 days. In almost every instance the bones

TABLE I.
Protective Experiments with Sunlight.

Rat No.	Diet.	Weight.		Sunlight exposure.		Rickets.											
		Initial.	Final.	Length of time daily.	Total.	Radiogram.	Pathological examination.										
							Gross.	Microscopic.									
246	No. 84	gm.	gm.	None (darkness).	hrs.	Rickets.	Rickets.	Rickets.									
247		30	32						"	"							
248		30	30						"	"							
436		20	24						Rickets.*	Rickets.	Rickets.						
437		40	50									"	"				
438		44	50	"	"	"	"	"									
		50	60														
249	No. 84	44	54	15 min. (24 days).	6	Negative.	Negative.	Negative.									
250		36	40						"	"	"	"	"				
251		30	32						"	"	"	"	"				
439		42	52						"	"	"	"	"				
440		40	52						"	"	"	"	"				
441		50	56						"	"	"	"	"				
442		44	50						"	"	"	"	"				
262	No. 84 + 25 mg. of K ₂ HPO ₄ .	54	70	None (darkness).		Rickets.	Rickets.	Rickets.									
263		52	72						"	"	"	"	"				
264		44	64						"	"	"	"	"				
443		30	40						Rickets.*	Slight.	Slight.	Slight.	Slight.				
444		34	48											"	"	"	"
445		34	50											"	"	"	"

259	No. 84 + 25 mg. of K_2HPO_4 .	40	60	15 min. (27 days).	8	Negative.	Negative.	Negative.
260		48	66			"	"	"
261		50	76			"	"	"
121	No. 84 + 75 mg. of K_2HPO_4 .	40	45	None (darkness).		Negative.	Negative.	Negative.
122		32	50			"	"	"
123		26	44			"	"	"
449		30	40			Rickets,	Slight.	Very slight.
450		34	46			Slight (?),	Negative.	Negative.
451		32	40			"	"	"
124	No. 84 + 75 mg. of K_2HPO_4 .	36	52	Indefinite.		Negative.	Negative.	Negative.
125		36	36			"	"	"

* Animals not killed; used for curative experiment.

were subjected to microscopic examination, and the final criterion as to the presence of rickets was the histological, rather than the radiographic picture.

Experiments with sunlight are difficult to carry out satisfactorily on account of the variability in the degree of sunshine, which makes exact quantitative work impossible. Nevertheless, as shown in Table I, daily exposures of 15 minutes sufficed to protect rats, fed a standard rickets-producing diet, which was adequate in its calcium but inadequate in its phosphorus content. This diet contained about 86 mg. of phosphorus per 100 gm. of diet; the addition of about 75 mg. of phosphorus to this diet is required to protect rats from rickets (Diet 85). The effect of sunlight may, therefore, be stated to have been equivalent to about doubling the quota of phosphorus. In considering the qualitative variability of the sun, it was noted in two series of experiments, not included in the tables, that the same degree of protection could not be obtained during November and December in rats on this diet. No doubt this is to be ascribed to the comparative lack of ultra-violet radiation furnished by the sun in the temperate zone at this season of the year, and is a factor to be considered in employing heliotherapy in infantile rickets.

As stated elsewhere, the potency of the sun is lost after its rays have traversed window glass (3). This observation is confirmed by experiments on rats. In the tests which are shown in Table II flint glass, 3.5 mm. in thickness, was employed. In another experiment window glass, 4.1 mm. in thickness, was employed with radiations from the mercury vapor lamp, as well as from the carbon arc lamp, for periods of 15 minutes. In both of these tests the animals developed marked rickets. A spectrogram of the second glass filter,¹ demonstrated that it obstructed rays shorter than 334μ in length.

Another experiment was devised so that the sunlight was reflected from a smooth white surface and did not impinge directly on the rats. It will be noted (Table II) that this arrangement permitted access of a certain amount of effective radiation to the animals, an amount not adequate to protect them, but sufficient to prevent the development of the severe grade of rickets which came about either in darkness or when window glass was interposed.

¹ This was supplied by the Corning Glass Works, Corning, N. Y.

TABLE II.
*Protective Experiments with Sunlight,
 Direct, Transmitted, and Reflected Light.*

Rat No.	Diet.	Weight.		Sunlight exposure.		Rickets.			Inorganic P per 100 cc. of blood.
		Initial.	Final.	Length of time daily.	Total.	Radiogram.	Pathological examination.		
							Gross.	Microscopic.	
528 530	No. 84	gm. 56 48	gm. 70 60	None (darkness).	hrs.	Rickets. "	Rickets. "	Moderate. "	mg. 5.2 4.9
535 536 524	No. 84	60 60 48	72 68 54	Direct. 30 min. (12 days), 60 " (14 ").	20	Negative. " "	Negative. " "	Negative. " "	
532 533 534	No. 84	60 52 58	70 54 60	Transmitted. 30 min. (12 days), 60 " (14 ").	20	Rickets. " "	Rickets. " "	Moderate. " "	3.75 3.11
525 529 527	No. 84	40 50 56	60 70 72	Reflected. 30 min. (12 days), 60 " (14 ").	20	Rickets. " "	Rickets. " "	Slight. " "	3.0 3.0 2.5

Experiments with the Mercury Vapor Quartz Lamp.

The rays from the mercury vapor lamp have been used successfully in infantile rickets by Huldschinsky (4) and others. They have also been employed in experimental rickets by Powers, Park, and their associates (5), and by Hess, Unger, and Pappenheimer (3). The rays of this lamp are referred to sometimes as artificial sunlight, but differ markedly from those of the sun in that their spectrum is linear and not continuous, and they do not extend so far in the region of the infra-red but much further in the region of the ultra-violet. Table III demonstrates that they are capable of affording marked protection against rickets when given in small amount. It is not necessary to treat the animals with the rays for hours, as has been done in previous experiments. With an alternating current of 160 volts, exposures of 3 minutes or less, at a distance of 3 feet, were found quite sufficient to accomplish this result. It may be noted also that these animals failed to develop rickets in spite of the fact that the content of inorganic phosphate of their blood was far below the normal. In many instances, as reported by Gutman and Franz (6), only 4 mg. was obtained per 100 cc. of blood, whereas the normal content is frequently as high as 8 or 9 mg., when measured according to the method of Bell and Doisy. In rachitic rats which were kept in the dark and fed this diet, these investigators found the average inorganic phosphate to be 3.2 mg. per 100 cc. of blood. Kramer and Howland (7) described a greatly diminished concentration of inorganic phosphate in rats rendered rachitic by a diet deficient in phosphorus and in the organic factor contained in fats.

An attempt was made to protect rats from rickets by irradiation before they were placed on the experimental diet. To this end a series of animals was rayed for 6 minutes daily, at a distance of 3 feet, for a period of 5 days. It was found, however, that this preliminary irradiation did not delay the onset of the rachitic lesions or decrease their intensity, so that it may be inferred that this treatment did not enable the animals to store or mobilize any protective substance in their tissues.

There are at least three factors which determine the effect of light—the diet, the rate of growth, and the degree of pigmentation of the

skin. The question of diet and rate of growth will be referred to in the discussion of subsequent experiments. The importance of the relation of skin pigment can be demonstrated by a simple experiment (Table IV). If two groups of rats, one composed of white rats and the other of black rats (the melanotic form of the Norway rat), are given the minimal protective dose of light, it will be found that although diet and rate of growth have been the same, the black rats will develop rickets, whereas the white rats will show no rachitic lesions (Figs. 1 to 3). The table presents the results of radiographs and gross and microscopic examinations of two groups of rats subjected to a test of this kind. It is manifest that the protective rays were rendered comparatively inert by the integumentary pigment.² The applicability of this experiment to infantile rickets, especially to the well recognized susceptibility of the negro infant, is so evident as to require no further comment.

Experiments with the Carbon Arc Lamp.

A large number of experiments has been carried out with the carbon arc lamp. In view of the fact that its spectrum resembles sunlight more closely than that of the mercury vapor lamp, it seemed as if this source of light might be of value in rickets. This was found to be the case both in experimental and in infantile rickets. By means of this therapeutic agent rickets in infants can be readily cured, and the cure is accompanied by a surprisingly rapid augmentation of the inorganic phosphate of the blood (8). In a large series of young rats it was found that daily exposures of 3 minutes, at a distance of 3 feet, regularly prevented the occurrence of rickets, and that 2 minute exposures sufficed frequently under these conditions. When the animals were irradiated only every other day, slight rickets developed (Table V). Furthermore, treating with the rays at a distance of 6 feet for 1 hour afforded protection, although at a distance of 9 feet the same period of exposure proved inadequate.

² In one experiment (Table IV, Rats 589, 592, 593, 594, and 597) a somewhat larger amount of irradiation was given and protection followed. This shows that the pigment is not to be regarded as an absolute filter of ultra-violet rays.

TABLE III.
Protective Experiments with the Mercury Vapor Quartz Lamp.
Variations in Duration of Exposures.

Rat No.	Diet.	Weight.		Irradiation.		Radiogram.	Rickets.		Inorganic P per 100 cc. of blood.
		Initial.	Final.	Exposure.	Dis- tance.		Gross.	Pathological examination.	
		gm.	gm.	min.	ft.			Microscopic.	mg.
528	No. 84	56	70	None (dark- ness).		Rickets.	Rickets.	Moderate.	3.07
530		48	60			"	"	"	3.0
598		88	90			"	Marked.	"	2.45
568		36	58			"	"	"	2.0
569		38	54						
726	No. 84	42	50	$\frac{1}{2}$	3	Rickets (?).	Slight.	Slight.	727 } 2.47
727		44	50			Negative.	Very slight.	" (osteoporosis).	729 }
728		42	44			"	"	Minimal.	3.0
729		40	44			Slight.	Slight.	Partial protection.	
722	No. 84	40	40	1	3	Negative. Rickets.	Very slight.	Slight (healing).	4.56
723		50	50				Slight.	"	3.95
724		52	64				"	"	
725		50	50				Very slight.	"	
872	No. 84	74	80	3	3	Negative.	Negative.	Negative.	4.5
873		50	50			"	"	"	4.2
874		70	64			"	"	"	
875		70	54			"	"	"	

730	No. 84	44	52	15	3	Negative. " " "	Negative. " " "	Negative. " " "	
731	42	50							
732	42	50							
733	44	50							
570	No. 84	40	54	$\frac{1}{2}$, 1, 1 $\frac{1}{2}$, 2	3	Negative. " " " (flattened out).	Very slight. " " Slight.	Negative. " " Minimal.	3.75 3.6
571	40	56							
599	90	92							
600	No. 84	90	95	1, 2, 3, 6	3	Negative. " " "	Negative. " " "	Negative. " " "	5.35 5.42
572	36	50							
573	40	58							
574	No. 84	38	50	2, 4, 6, 12	3	Negative. " " " (flattened out).	Negative. " " "	Negative. " " "	3.8* 4.34
575	38	98							
601			100						

* Test questionable.

TABLE IV.
*Protective Experiments with the Mercury Vapor Quartz Lamp.
White and Black Rats.*

Color of rat.	Rat No.	Diet.	Weight.		Irradiation.		Radiogram.	Rickets.		Inorganic P per 100 cc. of blood.
			Initial.	Final.	Exposure.	Dis- tance.		Gross.	Microscopic.	
White.	860	No. 84	gm.	gm.	min.	ft.	Negative. " "	Negative. " "	Negative. " "	mg.
	861		70	70	1	3				
	862		58 60 70	60 70						
White.	857	No. 84	60	80	1½	3	Negative. " "	Negative. " "	Negative. " "	5.45 4.44
	858		64	70						
	859		62	70						
Black.	854	No. 84	50	60	1	3	Rickets. Moderate. "	Marked. " "	Extreme. Rickets. "	
	855		50	60						
	856		60	58						
Black.	851	No. 84	50	50	1½	3	Moderate. Marked. " (fracture).	Marked. " Very marked.	Marked. " "	2.92 3.00
	852		48	54						
	853		60	60						
Black.	589	No. 84	90	100	½, 1, 1½, 2	3	Negative. " "	Very slight. " "	Negative. Very slight. Rickets.	4.11 3.22 2.90*
	592		48	70						
	593		50	56						
Black.	594	No. 84	84	100	1, 2, 3, 6	3	Negative. "	Negative. "	Negative. "	4.41
	597		36	48						

* Test questionable.

The protective effect of carbon arc light was tested also on rats fed more complex diets. Table VI shows that this source of light was effective when Diet 84 was amplified with serum albumin, egg albumin, or butter; in each instance a small amount of secondary potassium phosphate was added to compensate for the diminution of phosphorus incidental to replacing 5 or 10 per cent of the flour. Similar success was obtained with a diet amplified with dried milk. For some months experiments have been in progress with a diet containing dried milk, with the object of rendering Diet 84 more complete, and at the same time providing a food which more nearly resembles the dietary of the infant. It has been found that rickets will develop if, to a dietary composed of patent wheat flour and a simple salt mixture, about 10 per cent of dried milk (desiccated by the roller process) is added. It may be found that a diet containing 5 to 10 per cent of dried milk will be of value in investigations of experimental rickets in rats. The animals which received this more complete food were protected from rickets by irradiations of 4 minutes. Moreover, they grew exceptionally well. Whereas on Diet 84 rats increase about 10 gm. in weight in the course of the experimental period, some of the animals on this diet increased 30 gm. during the 26 days which comprised the experiment (Table VI). The factor of growth is of great importance in a consideration of measures which protect against rickets, as rapid growth tends markedly to the development of rickets. Protection afforded while the rate of growth is rapid indicates, therefore, increased potency of a therapeutic agent.

As demonstrated by McCollum, Simmonds, Shipley, and Park (9), rickets may be induced in young rats, on a diet low in phosphorus and high in calcium, or, on the contrary, on a diet low in calcium and high in phosphorus. Diet 84 is the prototype of the former diet and, as shown above, fails to induce rickets when the animals are exposed daily to a few minutes irradiation with the carbon arc lamp. Diet 85C is the prototype of the low calcium and high phosphorus diet, and regularly brings about rickets, although of somewhat different type from a histological point of view. Rats maintained on this diet were protected by light as readily as those on the low phosphate ration (Table VII), a result which suggests that light is able to compensate,

TABLE V.
Protective Experiments with the Carbon Arc Light.
Variation in Duration and Distance of Exposures.

Rat No.	Diet.	Weight.		Irradiation.		Rickets.			Inorganic P per 100 cc. of blood.
		Initial.	Final.	Exposure.	Dis- tance.	Radiogram.	Pathological examination.		
							Gross.	Microscopic.	
698	No. 84	gm. 24	gm. 30	min. 1, 1 1/2	3	Slight. Moderate. Slight.	Rickets. " "	Slight. Marked. "	mg.
699		40	50						
700		44	54						
696	No. 84	44	58	1, 2, 3	3	Slight. "	Slight. "	Slight. "	
697		34	50						
1015	No. 84	54	64	3	3	Negative. " Slight.	Negative. " Very slight.	Negative. " Minimal.	
1016		30	30						
1017		60	70						
980	No. 84	50	70	4	3	Negative. " " "	Negative. " " "	Negative. " " " (slight osteoporosis).	
981		80	84						
982		60	62						
983		50	60						
900	No. 84	54	70	5	3	Negative. " " "	Negative. " " "	Negative. " " " (slight osteoporosis).	
893		80	80						
906		40	44						
907		44	50						

892	No. 84	80	84	10	3	Negative. " "	Negative. " Negative.	Negative. } " Negative (slight osteoporosis).	3.2
908		50	50						
901		40	30						
909		50	44						
803	No. 84	50	62	15	3	Negative. " " "	Negative. " " "	Negative (osteoporosis). Negative. } " " (osteoporosis).	5.4 4.76 4.44
804		44	58						
805		42	60						
806		56	64						
749	No. 84	30	40	60	3	Negative. " "	Negative. " "	Negative. " "	
750		34	42						
751		50	60						
752	No. 84	30	40	60	6	Negative. " " (?)	Negative. " " (?)	Negative. " Minimal.	
753		30	38						
754		54	60						
759	No. 84	22	24	60	9	Slight. Moderate. " Slight.	Slight. " Moderate. Slight.	Slight. Moderate. " Slight.	
760		28	38						
761		24	30						
762		20	30						
1125	No. 84	30	40	5 (every other day).	3	Moderate. " Marked.	Marked. Slight. Marked. Slight.	Marked. Slight. Marked. Slight.	
1126		50	56						
1127		50	54						
1128		50	60						

TABLE VI.
Protective Experiments with the Carbon Arc Light.
Effect of Diet.

Rat No.	Diet.	Weight.		Irradiation.		Rickets.			Inorganic P per 100 cc. of Blood.
		Initial.	Final.	Exposure.	Distance.	Radiogram.	Pathological examination.		
							Gross.	Microscopic.	
1015	No. 84	gm.	gm.	min.	ft.				mg.
1016		54	64	3	3	Negative.	Negative.	Negative.	
1017		30	30			"	"	"	
		60	70			Slight.	Very slight.	Minimal.	
980	No. 84	50	70	4	3	Negative.	Negative.	Negative.	
981		80	84			"	"	"	
982		60	62			"	"	"	
983		50	60			"	"	" (slight osteoporosis).	
996	No. 84 + 10 per cent serum albumin + 50 mg. of K ₂ HPO ₄ .	60	60	4	3	Negative.	Negative	Negative (osteoporosis).	5.0
997		68	70			"	"	"	
998		60	68			"	"	" (moderate osteoporosis).	6.6
999		60	70			"	"	Negative (osteoporosis).	
992	No. 84 + 5 per cent butter + 25 mg. of K ₂ HPO ₄ .	54	60	4	3	Negative.	Negative.	Negative.	5.4
993		60	64			"	"	"	
994		66	54			"	"	"	
995		70	82			"	"	Negative.	5.0

984	No. 84 + 10 per cent egg albumin + 50 mg. of K_2HPO_4 .	58	64	4	3	Negative. " Slight. "	Negative. " " Very slight.	Negative (slight osteoporosis). " Minimal (healing).	4.45 4.8 4.0
985		60	64						
987		60	88						
		40	60						
1018	7.2 per cent dry milk + 5 per cent No. 84	48	62	None.		Moderate. "	Marked. Moderate. "	Moderate. Slight. "	4.45 5.0
1019	salt mixture* + 87.8	54	74			Marked. "	"		
1020	per cent patent	50	70						
1021	wheat flour.	50	64						
1022	11 per cent dry milk +	50	70	None.		Moderate. "	Moderate. Marked. Moderate. "	Slight. " Moderate. Slight.	7.15 6.66 5.0
1023	5 per cent salt mixture + 84 per cent	60	90			"			
1024	patent wheat flour.	50	80			"			
1025		50	70			"			
1294	10 per cent dry milk +	64	100	4	3	Negative (?). " "	Very slight. " "	Negative. " "	
1295	5 per cent salt mixture + 85 per cent	30	60			(?). "	"		
1296	patent wheat flour.	38	64			(?). "	"		

* Calcium lactate..... 2.9 per cent.
 Sodium chloride..... 2.0 per cent.
 Ferric citrate..... 0.1 per cent.

TABLE VII.
*Protective Experiments with the Carbon Arc Light.
 High Phosphorus, Low Calcium Diet.*

Rat No.	Diet.	Weight.		Irradiation.		Radiogram.	Rickets.	
		Initial.	Final.	Exposure.	Distance.		Pathological examination.	
							Gross.	Microscopic.
975	No. 84	gm. 50	gm. 72	min. 15	ft. 3	Negative. "	Negative. "	Negative. "
976		62	67					
977	No. 85C*	47	50	15	3	Negative. "	Negative. "	Negative. Almost negative.
979		35	37					
419	No. 85C	40	36	None.		Negative (?). " " (?) " (?) " (?)		Rickets (atypical). " " " "
420		46	42					
421		52	50					
422		54	53					

* Patent flour..... 95.0 per cent.
 Sodium chloride..... 2.0 per cent.
 Potassium phosphate (secondary)..... 2.9 per cent.
 Ferric citrate..... 0.1 per cent.

to an important degree, for a dietary deficiency of either calcium or phosphorus.

It seemed worth while to ascertain whether variations of temperature exert an effect on the protective action of light. For many years some have attributed the low incidence of infantile rickets in southern countries and in the tropics to the favorable influence of heat. Furthermore, ordinary chemical reactions are markedly accelerated by an increase of temperature. The test was planned so as to bring about a range of temperature of about 10°C . In order to accomplish this, some groups of rats were treated with the rays at room temperature, whereas others were placed in cages superimposed on ice. By this means temperatures of 29° , 23° , and 18°C . were established. The exposures were made for the standard period of 3 minutes at a distance of 3 feet. As shown in Table VIII fully as great protection was afforded at 18°C . as at 29°C ., almost no rickets developing in groups of rats exposed at either temperature. The effect of lower or of higher temperatures was not tested.

CONCLUSION.

Young rats on a diet low in phosphorus can be protected from rickets by irradiations with sunlight for about 15 minutes daily. In the winter months, however, this degree of light was found insufficient. The effective rays of the sun, in the intensities studied, did not penetrate window glass. They manifested some protective value after reflection from a smooth white surface.

Irradiation of a few minutes with the rays of the mercury vapor lamp suffices to protect rats against rickets. This is true likewise of the rays from the carbon arc lamp. A standard protective dose of radiation can be formulated for rats on a standard diet.

Light is able to prevent the occurrence of rickets in rats fed a rickets-producing diet characterized either by a low phosphorus and high calcium content, or a high phosphorus and low calcium content.

Moderate variations in temperature do not alter the effective action of light rays. Pigmentation of the skin markedly lessens their effect, as demonstrated by the failure of a standard dose to protect black rats.

TABLE VIII.
Protective Experiments with the Carbon Arc Light.
Effect of Temperature.

Rat No.	Diet.	Weight.		Irradiation.		Tem- per- ature.	Rickets.			Inorganic P per 100 cc. of blood.
		Initial.	Final.	Expos- ure.	Dis- tance.		Radiogram.	Pathological examination.		
								Gross.	Microscopic.	
		gm.	gm.	min.	ft.	°C.				mg.
1015	No. 84	54	64	3	3	29	Negative.	Negative.	Negative.	
1016		30	30				"	"	"	
1017		60	70				Slight.	Very slight.	Minimal.	
980	No. 84	50	70	4	3	29	Negative.	Negative.	Negative.	
981		80	84				"	"	"	
982		60	62				"	"	"	
983		50	60				"		(slight osteoporosis).	
892	No. 84	80	84	10	3	29	Negative.	Negative.	Negative.	3.2
908		50	50				"	"	"	
901		40	30				"			
909		50	44				Negative.	Negative.	(slight osteoporosis).	
1075	No. 84	70	80	3	3	23	Negative.	Negative.	Negative.	
1076		60	64				"	"	"	
1077		50	68				"	"	"	
1078		30	38				"	(?).	Almost negative.	
1071	No. 84	40	54	4	3	23	Negative.	Negative.	Negative.	(slight osteoporosis).
1072		40	50				"	"	"	
1073		42	50				"	"	"	
1074		36	48				"	"	"	

902	No. 84	50	50	10	3	23	Negative. " "	Negative. " "	Negative. " "	4.0
903		44	60				" "	" "	} (osteoporosis).	3.65
904		50	50				" "	" "		
905		44	50				" "	" "		
1083	No. 84	50	50	3	3	18	Negative. " "	Negative. " "	Almost negative.	
1084		50	50				" "	" "	Negative.	
1085		48	54				Very slight.	Very slight.	Minimal (healing).	
1086		50	54				Negative.	" "	Almost negative.	
1079	No. 84	50	54	4	3	18	Negative (?). " "	Negative. " "	Negative.	
1080		50	40				" "	" "	(marked osteoporosis).	
1081		50	60				" "	" "	Almost negative.	
1082		50	50				" "	" "		
933	No. 84	50	50	10	3	18	Negative. " "	Negative. " "	Negative.	
934		50	50				" "	" "	(osteoporosis).	
935		48	50				" "	" "	" "	
936		50	50				" "	" "	" "	

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EXPLANATION OF PLATE 43.

FIG. 1. Rat 859 (white). 31 days on Diet 84. Daily exposure to the mercury vapor quartz lamp for $1\frac{1}{2}$ minutes at a 3 foot distance. Rib: The zone of proliferating cartilage is normal. The zone of preparatory calcification (Pr_1) averages four cells in depth; matrix calcified. Complete calcification of spongiosa (Sp) and cortex (Co). No visible osteoid. No rickets. Decalcified in Müller's fluid for 5 days. Hematoxylin-eosin.

FIG. 2. Rat 853 (black). 31 days on Diet 84. Daily exposure to the mercury vapor quartz lamp for $1\frac{1}{2}$ minutes at a 3 foot distance. Rib: The zone of preparatory calcification (Pr_1) is almost wholly free from calcium, and is greatly increased in depth and prolonged into the metaphysis. There is great excess of perichondral (Pc) and subchondral (Sc) osteoid. Marked rickets. Decalcified in Müller's fluid for 5 days. Hematoxylin-eosin.

FIG. 3, *a* and *b*. (*a*) Radiogram of the knee joint from a black rat (No. 853), showing marked rickets with fracture of the tibia. (*b*) Radiogram from a white rat (No. 859), showing a normal epiphyscal line.

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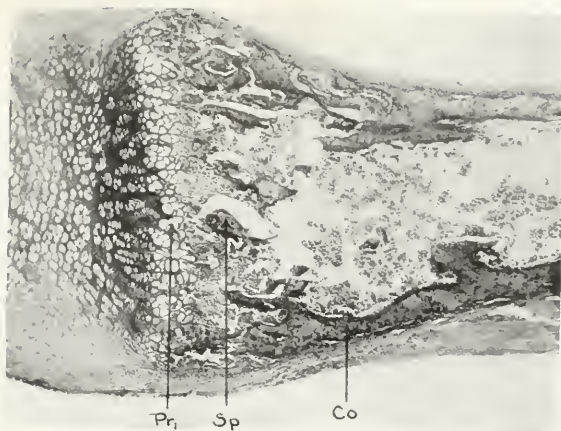


FIG. 1.

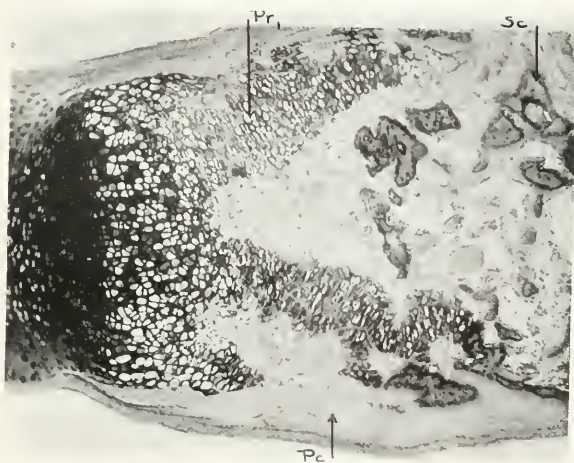


FIG. 2.



FIG. 3, a.

FIG. 3, b.

(Hess, Unger, and Pappenheimer: Experimental rickets. VII.)

EXPERIMENTAL RICKETS IN RATS.

VIII. THE EFFECT OF ROENTGEN RAYS.

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PLATES 44 AND 45.

(Received for publication, May 29, 1922.)

In view of the fact that the rays of the sun, of the mercury vapor lamp, and of the carbon arc lamp are able to protect rats from rickets, it seemed worth while to test the protective value of soft Roentgen rays. These experiments have been briefly referred to in another connection (1). As far as we are aware, the only report bearing on this subject is that of Huldschinsky (2), who treated a 3 year old rachitic child with rays from a tube having a hardness of 2.5-3° W., and observed calcification after eighteen irradiations in the course of 2 months. This author refers to a previous attempt at Roentgen ray therapy carried out by Winkler in 1920.

In addition to these preventive tests, a series of rats was subjected to massive doses of Roentgen rays, with the object possibly of damaging the cells to such an extent as to lead to the development of rickets. Many of the French writers, and especially Marfan (3) have held the opinion that changes in the bone marrow are of primary importance in the pathogenesis of rickets.

Table I shows the results of the protective experiments. The animals were on a rickets-producing diet, except that in one experiment 25 mg. of K_2HPO_4 per 100 gm. of diet were added in order to compensate for a supposed deficiency of phosphorus in the flour, and bring the diet up to the standard of 86 mg. per 100 gm. It will be noted that all the animals developed rickets, in spite of the irradiation, a result quite the contrary to that obtained when the mercury vapor or the carbon arc lamp was employed. This failure came about when the duration of the exposure was 15 minutes, and the distance of the

TABLE I.
Röntgen Ray Radiation. Attempted Protection from Rickets.

Rat No.	Diet.	Weight.		X-ray radiation.		Rickets.			Inorganic P per 100 cc. of blood.
		Initial.	Final.	Duration.	Type.	Radiogram.	Pathological examination.		
							Gross.	Microscopic.	
684	No. 84*	gm. 44	gm. 52	min. None (control).		Moderate. "	Rickets. "	Moderate. "	3.2
685		50	62						
678	No. 84	44	50	15	5 milliamperes, $\frac{3}{4}$ inch spark-gap, 8 inch distance, no filter.	Moderate. " " " " "	Rickets. "	Moderate. "	2.4
680		40	40						
679		40	40						
681		46	55						
683		34	40						
682		44	46						
795	No. 84 + 25 mg. of K_2HPO_4 .	52	54	2	15 milliamperes, $\frac{3}{4}$ inch spark-gap, 36 inch distance, † no filter.	Marked. " " "	Rickets. "	Moderate. "	3.2 3.33 3.5
796		52	54						
797		48	50						
798		56	62						

* Patent flour..... 95.0 per cent.
 Calcium lactate..... 2.9 per cent.
 Sodium chloride..... 2.0 per cent.
 Ferric citrate..... 0.1 per cent.
 † 18 inches the 1st week.

Coolidge tube 8 inches, as well as when the period was shortened to 2 minutes and the tube was placed at a distance of 3 feet. A $\frac{3}{4}$ inch spark-gap, 15 milliamperes, and a universal Coolidge tube without filtration were used. The radiographs of the epiphyses at the knee joint showed the changes typical of the rickets which comes about on this diet. This was true likewise of the histological sections. The cartilage was wide and irregular, excess of osteoid was present, and there was no evidence that the picture had been altered by the Roentgen ray exposures (Figs. 1 and 2).

A series of animals was next subjected to very large doses of Roentgen rays. For this purpose we employed a universal Coolidge tube, at 8 inches distance, with 3 mm. of aluminum as a filter, a spark-gap of 6 inches, and 5 milliamperes of current. The exposures were for periods of 3 and 6 minutes. The dietary was No. 85, which consists of No. 84 with 75 mg. of phosphorus per 100 gm. of diet added. This diet is just sufficient to prevent the occurrence of rickets, but contains little phosphate in excess, so that if the intensive irradiation tended to produce rickets, it should be rendered evident on this diet. In no instance, however, did rickets develop (Table II)—merely the osteoporosis which, in our experience, is regularly produced by Diet 85. Fig. 3 illustrates the histological structure of the rib of Rat 837, and as far as the bony and cartilaginous structures are concerned, presents the typical picture encountered on this diet. The protocol of this animal is given in Table III.

Of particular interest are the changes observed in the blood at the time of necropsy and the histological alteration of the bone marrow. There was marked anemia, a reduction of the red cells by fully one-half; the control rats on the same diet had 5,120,000 to 6,500,000 red cells per c.mm. whereas the rats treated with Roentgen rays had 1,500,000 to 2,500,000 red cells. The hemoglobin averaged about 37 per cent in contrast to 79 per cent in the control animals. One of the striking features was the leucopenia which came about; the total number of leucocytes of the four rats was, respectively, 2,600, 2,100, 1,000, and 1,600 compared with an average of about 12,000 in the control rats. The diminution was most marked in the lymphocytes. This lymphopenia, following large doses of Roentgen rays, has been emphasized by Murphy and Morton (4) and their associates, who

TABLE II.
Röntgen Ray Radiation. Attempted Production of Rickets.

Rat No.	Diet.	Weight.		X-ray radiation.		Rickets.			Inorganic P per 100 cc. of blood.
		Initial.	Final.	Duration.	Type.	Radiogram.	Pathological examination.		
							Gross.	Microscopic.	
838	No. 85*	gm. 46	gm. 38	min. 6	5 milliamperes, 6 inch spark-gap, 8 inch distance, 3 mm. aluminum filter.	Negative.	Negative.	Negative (osteoporosis).	mg.
839		40	50			"	"	"	
840		50	50			"	"	"	
841		44	40			"	"	"	
834	No. 85	46	58	3	5 milliamperes, 6 inch spark-gap, 8 inch distance, 3 mm. aluminum filter.	Negative.	Negative.	Negative.	4.36
835		40	50			"	"	"	
836		50	58			"	"	"	
837		50	40			"	"	"	
831	No. 85	36	40	None (control).		Negative.	Negative.	Negative.	4.26
832		42	48			"	"	"	
833		36	60			"	"	"	

* Diet 85 is No. 84 plus 75 mg. of P added as K_2HPO_4 .

found it to be the first change following radiation by Roentgen rays or by radium. The inorganic phosphate of the blood, 4.26 to 4.36 mg. per 100 cc., is approximately the percentage usually obtained in rats on this diet.

The bones showed no rachitic changes by radiograph during life, or by gross or microscopic examination after death. Fig. 3 illustrates the costochondral junction of Rat 837. It will be seen that it shows

TABLE III.
Protocol of Rat 837.

Date.	Weight.	Diet.	Remarks.
1921	gm.		
Dec. 17	50	No. 85	3 min. irradiation at 8 inches with filters.
" 19	50		
" 23	40		No irradiation Dec. 20 to 22, 24, and 26.
" 26	40		
" 30	44		
1922			
Jan. 3	54		No irradiation Jan. 1 and 2.
" 9	44		Jan. 10. Radiograph of epiphyses negative.
" 13	40		
" 16	40		
" 19			Killed for necropsy. Inorganic P of blood 4.60 mg. per 100 cc. (pooled with Rat 836). Hemoglobin 25 per cent; erythrocytes 1,250,000; polynuclear leucocytes 40 per cent; small mononuclears 52 per cent; large mononuclears 8 per cent.

none of the changes associated with rickets; the rows of cartilage cells are not increased in number, the matrix is well calcified, and there is no increase in osteoid tissue. The high power photograph (Fig. 4) is of especial interest. Here we note a marked alteration of the marrow. The specific marrow elements are replaced largely by edematous fatty tissue, containing cells resembling pale fibroblasts. Mitotic figures are occasionally seen in these cells. In other words, there is evidence of extreme exhaustion of the marrow.

CONCLUSION.

Rats on a low phosphorus diet cannot be protected from the development of rickets by exposures to soft Roentgen rays such as were employed in this study.

Furthermore, rats on a diet containing phosphate in an amount adequate to prevent rickets were not rendered rachitic by exposure to massive doses of Roentgen rays of an intensity sufficient to produce marked destruction of the blood-forming cells of the marrow.

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EXPLANATION OF PLATES.

PLATE 44.

FIG. 1. Rat 795. Diet 84 plus 25 mg. of K_2HPO_4 per 100 gm. of diet. 21 days on diet. Daily exposure to Roentgen rays (see Table I). Rib: Marked rickets. There is no evidence of prevention or healing under the influence of the Roentgen ray treatment. The greatly widened zone of preparatory calcification (Pr_1) is free from calcium in all portions. There is no recent calcium deposition in the perichondral osteoid (Pc) or in the osteoid of spongiosa (Sp_1) or cortex (Co). Decalcified in Müller's fluid for 5 days. Silver-nitrate-safranine.

FIG. 2. Rat 680. 21 days on Diet 84. Daily treatment with Roentgen rays (see Table I). No curative or preventive effect. The rib shows the usual typical rickets observed on this diet. Decalcified in Müller's fluid for 5 days. Silver-nitrate-safranine.

PLATE 45.

FIG. 3. Rat 837. 33 days on Diet 85. Intensive exposure to Roentgen rays (see Table II). Section of rib, low power. The zone of preparatory calcification (Pr_1) does not exceed five cells in depth. The matrix is densely calcified. The trabeculae of the primary spongiosa (Sp_1) are short and irregular, and not surrounded by well formed osteoblasts. The cortex (Co) is relatively thick, and a distinct osteoid border is not visible, either about the cortex or the spongiosa. The marrow (M) is almost depleted of blood-forming elements, being composed of a loose edematous fibrous and adipose tissue, with congested blood vessels. Decalcified in Müller's fluid for 5 days. Hematoxylin-eosin.

FIG. 4. Rat 837. Bone marrow, showing depletion of blood-forming cells. $\times 420$.

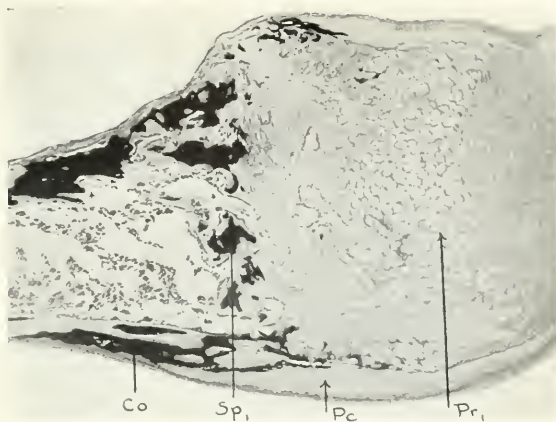


FIG. 1.



FIG. 2.

(Hess, Unger, and Steiner: Experimental rickets. VIII.)

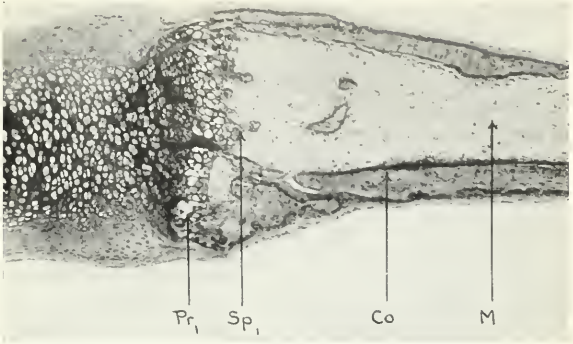


FIG. 3.

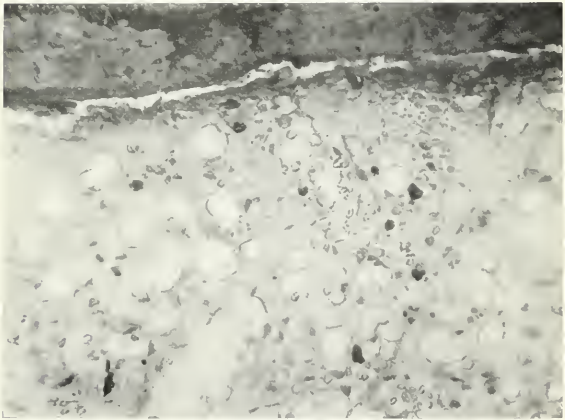


FIG. 4.

(Hess, Unger, and Steiner: Experimental rickets. VIII.)

COW SERUM AS A SUBSTITUTE FOR COLOSTRUM IN NEW-BORN CALVES.

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In a former communication¹ it was shown that if new-born calves are divided in two groups, one of which receives colostrum from the dam and the other not, and if both groups are removed promptly from the surroundings of the herd, placed in individual stalls, and fed raw milk, the calves fed colostrum survive and develop as normal calves, while of the calves from which colostrum has been withheld the greater number die within the 1st week as a result of the invasion and multiplication of intestinal bacteria, chiefly *Bacillus coli*, throughout the body. Those that survive may develop lesions in or about certain joints, more rarely foci of sclerosis in both kidneys.

EXPERIMENTAL.

The rapid absorption into the blood of agglutinins towards *Bacillus abortus* ingested in the colostrum² indicated that the immunity of the calves receiving colostrum is due to the protective antibodies which tend to accumulate in the colostrum up to the time of parturition. If this inference is true the blood of the adult cow should also contain these various antibodies and might protect calves not fortified by colostrum. To test this hypothesis, three groups of calves were treated with the blood serum of the same cow in slightly different ways.

Calves of which it was certain that they had not suckled the dam were brought to the animal quarter of the Department soon after birth, thoroughly washed with water of about body temperature, and

¹ Smith, T., and Little, R. B., *J. Exp. Med.*, 1922, xxxvi, 181.

² Little, R. B., and Orcutt, M. L., *J. Exp. Med.*, 1922, xxxv, 161.

dried by rubbing with towels. Each calf was placed in a separate stall and fed milk from cows in about the middle of the lactation period. The milk was at first fed from bottles or other receptacles through nipples and after the 1st week from pails.

The serum used throughout was obtained from the same cow at intervals during the experiment. The animal was a grade Guernsey, in milk when purchased from the Middle West November, 1920. She was chosen at random mainly because easily controlled. The blood was drawn from a jugular vein under aseptic conditions and allowed to clot. The serum was withdrawn after 2 days, passed through a Berkefeld filter, and stored at 38–40°F. without an antiseptic.

No efforts beyond isolation in individual stalls and ordinary cleanliness were made in rearing the calves. Owing to the nature of the experiment and the many uncontrolled factors entering into it, it has seemed best to record certain details which may be of significance in the interpretation of results. These are given in the notes on the individual calves.

The first group of five calves received serum both into the jugular vein and the subcutis immediately after the cleansing bath. On the following day serum was again injected into the subcutis. The following notes furnish the chief details.

Calf 901.—Guernsey bull, born Mar. 10, 1.15 p.m., of a native cow in her second pregnancy. Removed from dam immediately after birth. Received at Department 2.45 p.m. Washed in water at 100°F. containing a little soda. Dried off by gentle friction with bath towels. Calf quite weak after the cleansing. Kept warm with blankets. Receives into jugular vein 20 cc. serum and 20 cc. into subcutis in three different places. 5 p.m. Fed 1½ lbs. milk. 11 p.m. Second feeding of ¾ lb. milk.

Mar. 11. Meconium discharged during the night. Calf received about 2 lbs. milk. Urine collected has a specific gravity of 1,008. Contains no abnormal products. At noon calf received into subcutis 20 cc. serum. Diarrhea started late today. Temperature normal. Total amount of milk taken about 4 lbs.

Mar. 12. Stools less fluid, light yellow. Temperature normal. Total milk taken about 6 lbs.

Mar. 13. Feces consistent, orange to yellow. 7½ lbs. of milk taken.

Mar. 16. Calf is fed from pail. Takes about 7½ lbs. of milk.

Apr. 12. Calf has progressed normally, taking slightly more milk each week. Today 12 lbs. consumed. Since Apr. 4 and 5 a little hay and grain were placed in the stall each day.

Apr. 13. Calf killed today. Weight 102 lbs. Autopsy negative with exception of cecum in which there is a wide meshed pigmentation over the submucous vessels.

Calf 538.—Holstein bull, born 12.40 p.m., Mar. 16, of a cow in her third pregnancy. Brought to Department at 1.15 p.m. Washed in the same way as described for Calf 901. 2 p.m. Fed $\frac{3}{4}$ lb. of milk. 2.15 p.m. 18 cc. of serum injected into jugular vein and 22 cc. into subcutis. Fed milk at 5 and 10.30 p.m. Calf weak and able to stand only a short time.

Mar. 17. Begins to discharge meconium during the day. Takes milk from bottle readily, about 4 lbs. in all taken today. Stronger, moves about stall. Receives 20 cc. serum into subcutis at 3.30 p.m. Late in the evening calf was quite weak, scarcely able to stand. Extremities cold. Mucous membranes pale. Able to take only a little food at 11 p.m.

Mar. 18. Dies at 2.30 a.m. Weight after death 66 lbs. The autopsy showed no gross lesions to account for death. The fourth stomach contained over a liter of milky fluid and curds. The latter made up about one-third of the total. Mucosa was sprinkled over with deep and superficial petechiæ.³ In the small intestine occasional congested areas. In the large intestine the longitudinal folds form dark red streaks of congestion and hemorrhage. Cultures containing bits of tissue from spleen, liver, and kidneys remain sterile. From several segments of the small intestine *B. coli* isolated as the chief type present.

Calf 485.—Guernsey heifer, born Mar. 24, 9.15 a.m., of a native cow in her third pregnancy. Removed immediately and brought to Department. Washed and dried. 10.27 a.m. Injected 20 cc. serum into jugular vein and 20 cc. into subcutis. Calf rather weak. 1.45 p.m. Receives the first dose of milk from bottle. Fed again at 5 and 11 p.m. Calf active. Rather dry meconium voided.

Mar. 25. Feces growing soft, liquid later in the day. In the evening the calf loses its activity and takes its milk more slowly. Feces very soft and somewhat fetid; light yellow in color. Took in all about 3 lbs. of milk.

Mar. 26. Has been scouring during the night and continues during the day. Fed five times today, the calf taking only small amounts. Very weak late at night.

Mar. 27. Calf very weak, unable to get up. Takes a few ounces of milk. Temperature 37.2°C. Extremities growing cold. Chloroformed later in the afternoon and refrigerated. Calf weight 49 lbs.

Mar. 28. Autopsy shows the following abnormalities. Fourth stomach nearly empty. Minute hemorrhages present in small number. Upper small intestine congested in streaks and patches. Fat stasis indicated by whitish club-shaped villi. Epithelium contains fat granules. The rest of small intestine deeply congested in streaks, patches, and continuously for some distance. Longitudinal streaks of congestion in large intestine. Kidneys uniformly congested. Lungs mottled with pale red patches of atelectasis.

³ Smith, T., and Little, R. B., *J. Exp. Med.*, 1922, xxxvi, 184.

Agar slant containing a bit of kidney tissue remains sterile. One plus spleen tissue develops only around the bit of tissue. Subcultures from this contain besides *B. coli* a small diplococcus (enterococcus). A tube containing a bit of liver tissue also develops growth only about the tissue. Subcultures show only *B. coli*. The same type of *B. coli* was isolated from two segments of the small intestine in pure culture. This type is characterized by marked viscosity of the growth and large involution forms. Occasional motile elements detected.

Calf 767.—Holstein heifer, first calf of native cow, born 11.15 a.m., Mar. 24. Removed at once from dam, taken to Department, washed, dried, and blanketed. 11.55 a.m. Receives 20 cc. serum into a jugular vein and 20 cc. into the subcutis. Fed first milk at 3.15 p.m., next at 11.10 p.m. Calf, weak after cleansing, was stronger at night.

Mar. 25. Voids a large amount of dark greenish meconium. At 11 a.m. 20 cc. serum injected into subcutis. Fed three times today, in all about 3 lbs. of milk.

Mar. 26. Feces becoming softer and fluid late in the day, yellowish in color. Fed three times, in all 3½ lbs. of milk.

Mar. 27. Weaker today. Feces still fluid. Took only 3½ lbs. of milk.

Mar. 28. Calf quite weak. Feces still soft and yellowish. Takes 3½ lbs. of milk in all.

Mar. 29. Calf unable to stand. Has to be held when taking food. Fed four times. Took 3½ lbs. in all.

Mar. 30. Calf begins to improve. Feces less fluid. Takes 4 lbs. of milk. Gets up and moves about stall.

Apr. 19. Since Mar. 30 the calf has improved steadily and continued to take larger quantities of milk. Today 11 lbs. taken. The calf is emaciated and shows stiffness, without localized joint lesions, in the hind limbs. For several days (Mar. 30 and 31) there was a slight conjunctivitis.

Apr. 20. Calf killed today. Weight 78 lbs. No lesions were found. Cultures made with bits of spleen, liver, kidneys, and several drops of fluid from both knee and tarsal joints remained sterile.

Calf 948.—Female Holstein calf, born of a heifer in her first pregnancy, at 1.30 p.m., May 3. Calf weighs 88½ lbs. Labor difficult and calf delivered in the last stage by traction. Removed at 3.50 p.m. to the Department, washed, and dried. 4.40 p.m. 40 cc. serum injected into jugular veins and 20 cc. into subcutis. 7.50 p.m. First meal of 2 lbs. of milk. Fed again at 10.30 p.m. and 1 a.m.

May 4. Receives into subcutis 40 cc. serum. Fed four times today, the last meal at 10 p.m. Drank in all about 8 lbs. of milk.

May 5. Feces becoming soft and observed to be very watery and ejected in a stream at 1.15 p.m. Calf weak, growing thin. Eyes sunken. Has taken only about 6 lbs. of milk today.

May 6. Calf weaker; still able to stand but gait unsteady. Feces fluid during the day, light yellow in color. Takes in all about 4 lbs. of milk.

May 7. Temperature is 40.2°C. this morning. Feces continue fluid. Calf unable to stand. Takes but little milk when fed at 11 a.m. 48 cc. of cow serum injected subcutaneously. Calf dies this afternoon. Body chilled with cold water and refrigerated till next morning.

May 8. From the autopsy notes the following may be mentioned. The fourth stomach contains a small amount of a thick, viscid material plus small curds. Hemorrhagic blotches scattered through leafy portion. In the small intestine there is moderate congestion in patches above which increases in intensity downwards. In the ileum the mucosa is dark red and overlaid with a surface film of orange-colored mucoid material suggesting superficial necrosis. The large intestine is congested along summits of the longitudinal folds. Films from small intestine contain many rods, like *B. coli*.

Bits of spleen and kidney in agar slants remain sterile. In the liver tube one colony of *B. coli* appears. The dilution cultures from the small intestine contain colonies of *B. coli* only.

It will be noted that two of the five calves died in about 48 hours after birth. A third was chloroformed, moribund, when 3½ days old. A fourth calf was very sick when 3 days old, but recovered. The fifth calf passed through a period of diarrhea lasting nearly 2 days, but continued to take its food and made a good recovery.

Of the three calves that died, the organs of one were sterile. In the second case, the kidneys were sterile but growth appeared about the bit of liver and spleen tissue, indicating the presence of very few bacteria as compared with the condition of the same organs in controls not receiving either colostrum or serum. In the third both spleen and kidneys were sterile and the liver tube contained one colony.

A second group of five calves received the bovine serum not only intravenously and subcutaneously but also in the milk of the first two meals. The following notes of these cases supply the individual data.

Calf 768.—Holstein bull, born 5.45 p.m., Apr. 10, of a native cow in her third pregnancy. Weighs between 85 and 90 lbs. Removed immediately to the Department, washed, and dried. 7.20 p.m. 20 cc. serum injected into subcutis and 20 cc. into jugular vein. 10.10 p.m. Fed nearly 2 lbs. of milk plus 60 cc. serum mixed with it. 2.30 a.m. Fed milk and 60 cc. serum.

Apr. 11. Considerable meconium passed during the night. 20 cc. serum injected subcutaneously at 9.45 a.m. Takes in all 8 lbs. of milk today.

Apr. 12. Diarrhea during night and this morning. Takes food as usual.

Apr. 13. Feces soft and grayish yellow. Calf takes 8 lbs. of milk today.

Apr. 30. The calf has been progressing normally since the preceding date. Took 11 lbs. of milk today.

May 1. Killed and found normal.

Calf 905.—Holstein bull, born Apr. 7, at 10.30 a.m., of a native cow in her fourth pregnancy. Weighs 114 lbs. Taken to the Department immediately after birth, washed, and dried. 11.40 a.m. 20 cc. serum injected into a jugular vein and 20 cc. into subcutis. 2 p.m. Received first meal of milk, $1\frac{1}{2}$ lbs. plus 60 cc. serum. 5.40 p.m. Fed the same quantity of milk and serum. 10.35 p.m. Fed 2 lbs. of milk.

Apr. 8. Receives 20 cc. serum into subcutis at 10 a.m. Considerable dark greenish meconium discharged today. Feces soft late at night.

Apr. 9. Feces still fluid. Considerable blood discharged at one time during defecation. Calf otherwise active and hungry. Takes in all today 9 lbs. of milk.

Apr. 11. Feces still slightly tinged with blood.

Apr. 13. Takes in all 11 lbs. of milk. Lameness in right fore leg without local signs.

Apr. 14. Lameness in right hind leg. Hock and ankle swollen. Apr. 15. Temperature slightly elevated ($39.6^{\circ}\text{C}.$). Blood still voided on feces. Apr. 17. Stiffness and lameness have disappeared. Calf very active. Apr. 23. Takes 12 lbs. milk. Apr. 28. Slight febrile disturbance. Apr. 29. Normal today.

May 1. Killed for food. Organs normal.

Calf 906.—Holstein female, born 9.30 a.m., Apr. 11, first calf of a native heifer. Calf small, weighing 65 lbs. Received at Department at 10.30 a.m., washed, and dried. At 11.10 a.m. 20 cc. serum injected into a jugular vein and 20 cc. into the subcutis. 2 p.m. Fed 2 lbs. of milk to which were added 60 cc. serum. 5.35 p.m. The same dose repeated. 10.30 p.m. Fed 2 lbs. of milk.

Apr. 12. Receives into subcutis 20 cc. serum. Meconium passed during the night. Calf active and takes its food well.

Apr. 13. Feces liquid, greenish yellow. Calf otherwise normal. Apr. 15. Took $6\frac{1}{2}$ lbs. of milk today. Apr. 18. Took 8 lbs. of milk today. Apr. 21. Took 11 lbs. of milk today.

May 1. Killed for food. Organs normal.

Calf 932.—Holstein bull, born of a western cow, at 7.20 a.m., Apr. 17. Calf weighs 100 lbs. Taken to Department, and washed and dried. 10 a.m. Receives 20 cc. serum into a jugular vein and 20 cc. into the subcutis. Refuses food at noon. Takes 2 lbs. of milk at 4.50 p.m. to which 60 cc. serum had been added. At 10.15 p.m. this dose was repeated.

Apr. 18. Receives 20 cc. serum into subcutis. Calf constipated.

Apr. 19. Feces fluid, light yellow in color, later in day nearly colorless. Takes milk normally.

Apr. 20. Feces still soft.

Apr. 21. Takes 9 lbs. of milk in all. Some blood discharged on feces.

Apr. 24. Takes 11 lbs. of milk. Appears entirely normal. Apr. 26. Took 11 lbs. of milk today. Some blood passed on feces. Apr. 27. Blood still passed with feces. Apr. 29. Feces normal.

May 1. Killed for food. Normal.

Calf 953.—Guernsey female, born May 20, 9.15 a.m., of a cow in her fourth pregnancy. Calf rather thin, weighs 70 lbs. Received at the Department at 10.15 a.m. Simply dried off, without preliminary washing, with bath towels. Calf able to stand and move about. 10.40 a.m. Received into jugular vein about 39½ cc. of serum and 20 cc. into subcutis. 11 a.m. Fed 2 lbs. of milk plus 55 cc. serum. 4.20 p.m. The same feeding repeated. 11.15 p.m. Fed 2 lbs. of milk.

May 21. Calf very eager for food. 2 lbs. of milk taken. 9.30 a.m. 20 cc. serum injected into subcutis. Urine collected at this time. No fluid feces observed today. Fed in all 6 lbs. of milk.

May 22. Took 6½ lbs. of milk today. Feces passed resemble meconium. No diarrhea.

May 26. Slight diarrhea today. For several days past, the feces have been rather light in color but of normal consistency. Today the calf took 8 lbs. of milk.

May 27. Feces slightly softer than normal, light yellow. Urine collected early shows 0.06 per cent albumin (Esbach), specific gravity 1,010, slightly acid, pale yellowish, and faintly clouded. Sediment slight, containing some short hyaline casts filled with amorphous granules.

May 28. Feces normal. Only a trace of albumin in urine today.

May 30. Normal.

Two of these five calves manifested a slight diarrhea associated with hemorrhage which soon disappeared. Four were killed when 14, 21, 24, and 20 days old respectively. No abnormalities were noted. The fifth is still alive.

A third group of five calves received the serum in the milk of the first and second meals. Whatever protection was afforded by it had to be exerted through the digestive tract.

Calf 772.—Small Guernsey calf, born at 12.10 p.m., Apr. 19, of a native heifer in her first pregnancy. Weight 50 lbs. Taken away before it had suckled the dam, received and cleansed at 2 p.m. Calf very weak, unable to stand. Large amount of dark, soft meconium voided. 4.45 p.m. First feeding of 1 lb. of milk to which 100 cc. of serum had been added. 10.30 p.m. Calf fed 1½ lbs. of milk to which 100 cc. of serum had been added. Still unable to get up.

Apr. 20. Calf continues weak. Fed milk at four intervals during the day. During the last feeding at 10.15 p.m. the calf failed to take its food. Meconium voided during the day, now yellowish.

Apr. 21. Calf died at 4 a.m. Considerable diarrhea preceding death. Autopsy next morning presents the following abnormalities.

Fourth stomach contains a handful of white, rather firm curds and some milky fluid. Mucosa normal. Mucosa of small intestine deeply congested throughout. Large intestine less so.

Endocardium of left ventricle raised in the form of linear ridges by subendocardial hemorrhages.

Kidneys deeply and uniformly congested throughout. Urine taken from bladder shows only a trace of clouding when slightly acidified with acetic acid and heated to boiling.

Cultures prepared by adding bits of tissue from spleen, liver, and kidneys to slanted agar remained sterile. Cultures from successive dilutions of contents of small intestine contain colonies of *B. coli* and some colonies of smaller size, probably enterococci.

Calf 935.—Holstein female, weighing about 85 lbs., born 6.30 a.m., Apr. 21, of a heifer. Calf not allowed to suckle dam. Received at Department at 9.40 a.m., washed, dried, and blanketed. 11.40 a.m. Received first meal of 2 lbs. of milk plus 100 cc. serum. 4.30 p.m. The same dose repeated. 10.20 p.m. Fed 2 lbs. of milk.

Apr. 22. Dark meconium passed during night. Feces grew liquid towards noon, yellowish in color. Calf took $4\frac{1}{2}$ lbs. of milk in all today.

Apr. 23. Scouring a little early in the day. No movements later. Fed four times and took about 7 lbs. of milk.

Apr. 24. Calf improving and quite active. Fed three times, in all about 7 lbs. of milk. Urine caught is normal, specific gravity 1.012.

May 15. Has been taking 15 lbs. of milk daily. In good condition. Sold to butcher today. The organs appear normal with exception of kidneys. On close scrutiny there are seen in many of the lobes groups of feebly accentuated grayish areas less than 1 mm. in diameter. On section these correspond to narrowly triangular pale areas passing radially to medulla.

Calf 936.—Large Holstein bull, weighing about 100 lbs., born 9.20 a.m., Apr. 21, of a heifer (first pregnancy). Owing to its large size calf had to be delivered by traction in the last stage. Calf kept from suckling dam and taken to Department at 11 a.m., washed, dried, and blanketed. Milk refused by calf until 4.35 p.m. when it took $1\frac{3}{4}$ lbs. plus 100 cc. serum. A little of this mixture was lost in feeding. 10 p.m. Fed about 2 lbs. of milk plus 100 cc. serum. The calf has been rather weak all day.

Apr. 22. Large amount of meconium passed during the night. Feces becoming fluid during the day. Takes but little milk.

Apr. 23. Calf more active and feces firmer, yellowish in color. Fed four times, taking in all about 8 lbs. of milk.

Apr. 24. Calf active. Feces still soft and light yellowish in color.

Apr. 29. Slight swelling and lameness of left tarsal joint. Left fore leg shows lameness but no recognizable swelling. Receiving 12 lbs. of milk.

May 13. Calf not quite normal. Drank milk slowly. Evening temperature 39.9°C.

May 14. Condition unchanged. Takes 12 lbs. of milk. Temperature 39°, 39.8°, and 40°C.

May 15. Temperature 40°C. Killed today. The only abnormality found is in the kidneys. Multiple foci varying from 2 to 8 mm. in diameter; from two to twelve in a lobe. These are firm, smooth, and glistening white on section. They are broadly triangular with base at the surface and apex at junction of cortex with medulla. No reactive zone around them.

Calf 940.—Large Holstein heifer, weighing about 110 lbs., born 2.20 p.m., Apr. 25, of a native cow in her second pregnancy. Treated in the usual way. Calf weak and unable to stand. 4.40 p.m. Received the first food, 2 lbs. milk plus 100 cc. serum. 11 p.m. Fed 2½ lbs. milk plus 100 cc. serum. Dry meconium discharged.

Apr. 26. Feces became fluid towards afternoon, light yellow in color. Fed four times today. Took in all 8 lbs. of milk.

Apr. 27. Diarrhea checked. Calf active and hungry.

Apr. 29. Takes 11 lbs. of milk.

May 3. Takes 14 lbs of milk.

May 15. This calf has been normal, taking 17 lbs. of milk since May 11. Killed today. Organs normal.

Calf 951.—Holstein female, born May 8, at 4.30 p.m., of a heifer. Parturition normal. Calf weighs 77 lbs., rather thin. Taken to the Department at 5 p.m., washed, dried, and removed to a single stall. 7.10 p.m. Fed the first meal of milk plus 100 cc. cow serum. 11.40 p.m. Received 2 lbs. of milk plus 120 cc. serum. Moves actively about stall.

May 9. Fed 2 lbs. of milk plus 120 cc. serum. Calf takes the mixture readily. This is the last serum fed. Milk taken at 5 and 10 p.m. Calf has been constipated. Meconium passed during the night.

May 10, 8.15 a.m. Temperature slightly elevated (39.4°C.). Diarrhea sets in. Calf feeds slowly and shows signs of depression. Grows weaker during the day while diarrhea continues. Feces tinged with blood. Temperature at 11 p.m. 39.6°C.

May 11. Calf dies at 5 a.m. Chilled with cold water and refrigerated until 9.30 a.m. Of the autopsy notes, the following are significant. Fourth stomach contains a small amount of milky fluid and some curds, totalling a mass 4 to 5 cm. in diameter. Mucosa covered with a thin layer of mucus. Minute hemorrhagic points, deep, faded, and superficial appear in two dense groups covering a total area about 10 cm. in diameter. Small intestine congested throughout in streaks and patches, the congestion increasing downwards. Large intestine congested along longitudinal folds.

Medulla of kidneys deeply congested. Bladder distended with 775 cc. urine. No obstruction found to account for the retention. Specific gravity of urine 1,010. Faintest trace of albumin present.

Marked fatty degeneration of liver cells. Orange pigment granules present.

Bits of liver and kidney in agar tubes remain sterile. In the spleen tube in place of *B. coli* about fifteen colonies of an enterococcus present. *B. coli* appears in cultures from the small intestine as in earlier cases.

It will be noted that two of this group died. One was small and weak from the start, unable to get up, and lived less than 2 days. The spleen, liver, and kidneys were sterile, however. Since the organs of all control calves thus far contained numerous bacteria, it may be assumed that the serum was responsible for this condition.

The second calf which died lived $2\frac{1}{2}$ days. The liver and kidneys were sterile but in the spleen tube about fifteen colonies of enterococcus appeared.

Since in the former series¹ such a high percentage of calves from which colostrum had been withheld died, only two additional calves were treated in a similar way as controls during the period of this experiment. These died within 2 days. Spleen, liver, and kidneys were flooded with bacilli of the *Bacillus coli* type.

Calf 945.—Female, born of a native heifer Apr. 30, 10.20 a.m. Weighs $79\frac{1}{2}$ lbs. Not allowed to suckle the dam and taken to the Department buildings at 10.40 a.m. Washed and dried and removed to a single stall. Calf fed at 1 p.m. Drinks with difficulty and takes only 10 ounces. 6 p.m. Takes about 1 lb. of milk very slowly. Fed again about $1\frac{1}{2}$ lbs. at 10 p.m. During the day considerable meconium was discharged.

May 1. Takes about 8 lbs. of milk during the day. Bright and active early in the day. Feces become watery later in the morning and continue so during the day. Later tinged with blood.

May 2. Calf died during the night. Autopsied early this morning. Externally nothing abnormal. The fourth stomach somewhat dilated. It contains several firm white curds, the size of an egg, and considerable milky fluid. The entire mucosa has a deep red color. In the small intestine the mucosa is diffusely and deeply congested in the middle portion, in upper and lower segments less so. Films show many bacilli resembling *B. coli*.

Beneath endocardium of left ventricle there are many hemorrhages. The blood in general is in soft, dark clots, filling the large vessels with large molds. The liver contains much fat and orange pigment. The kidneys are uniformly congested to the tips of papillae. Urine from bladder contains a trace of albumin; clear, yellow, specific gravity 1.032.

Films from spleen, liver, and kidney tissue contain scattering colon-like rods in every field. Cultures from these organs indicate presence of large numbers of bacteria. Only after one dilution of a bit of tissue in 6 cc. of bouillon are colonies

discrete on agar slants. The bacteria resemble *B. coli*. Dilution cultures from a segment of the middle zone of the small intestine show *B. coli* and enterococci.

Calf 947.—Holstein bull calf, delivered by mechanical help, May 3, 10 a.m. Presentation normal. Mother a native heifer. Weight of calf 86 lbs. Taken to Department at 11.50 a.m. and treated as the others. It was noted when cleansing calf that the umbilical cord had been torn away during delivery. Raw surface treated with boric acid. 2 p.m. Received first meal of 2 lbs. of mixed milk. Fed again at 5.20 and 10.20 p.m. Received in all 6 lbs. of milk.

May 4. Calf's feces becoming fluid. Calf growing dull. Respirations rapid and superficial. Refuses midday meal. Fed at 5 and 10 p.m. Calf growing weaker, takes but little food. Discharges continue watery.

May 5. Feeding continued at intervals during the night and day. Symptoms as yesterday. Calf grows weaker, takes but little food. Dies towards evening. Refrigerated until next morning.

The autopsy showed few differences between this case and Calf 945. There were many superficial hemorrhagic points in the fourth stomach. The small intestine and the kidneys showed very little congestion. The liver contained considerable fat and orange pigment. The bacteriology of the organs and the small intestine was almost identical with that of Calf 945 and a description of the cultures is therefore superfluous.

During this same period two calves were placed under observation which had been permitted to take colostrum. One of these died after $5\frac{1}{2}$ days. This was the first colostrum-fed calf to die in our hands. The other developed normally after a short period of diarrhea. The dead calf's organs were sterile.

Calf 949.—Holstein bull calf, weighing 99 lbs., born of a native heifer, May 3, 5.15 p.m. Labor prolonged from 11 a.m. Presentation normal. Traction applied to fore legs in the last stage. Calf allowed to suckle dam for 15 minutes, then removed to Department, washed, dried, and bedded in clean stall.

May 4. Fed 8 lbs. of milk in four doses. Calf constipated.

May 5, 10.30 a.m. Large amount of meconium and yellowish feces passed. Diarrhea started in afternoon. Respirations accelerated. Temperature 39.6°C . at 5 p.m.

May 6. Feces continue fluid. Light yellow in color. Calf appears sick. Temperature 40.1°C . at 10 p.m. Has been taking food regularly until night.

May 7. Diarrhea continues through the day. Calf growing thin. Temperature 40.2°C . at night. Took about 7 lbs. of milk today.

May 8. Calf growing weaker and unable to stand up and swallow its food late at night. Diarrhea continues.

May 9. Calf died at 4.30 a.m. Refrigerated until 9.30 a.m. Weight 76 lbs. From the autopsy notes, the following conditions are noteworthy.

The fourth stomach was distended with about 2 liters of a milky fluid and few curds. The mucosa was overlaid with mucus and congested. The small intestine was slightly congested above. The congestion increased downward and was severe in the ileum. The large intestine was congested along summits of longitudinal folds. The other viscera showed nothing unusual.

Tubes of agar containing bits of spleen, liver, and kidney remain free from growth. In dilution cultures of the middle region of the small intestine, *B. coli* was present as in preceding cases.

Calf 769.—Holstein bull calf, born Mar. 29, 8.15 a.m., of a native cow in her second pregnancy. Calf weighs about 100 lbs. 10.30 a.m. Placed with dam and allowed to suckle. Removed to Department at 1.30 p.m., washed, dried, and placed in a fresh stall. The cord had been torn off close to the abdominal wall. The exposed area dusted with boric acid powder. Calf appears strong and able to move about. Fed at 10.30 p.m. $1\frac{1}{2}$ lbs. of milk.

Mar. 30. Feces become fluid during the day. Calf active. Takes in all $5\frac{1}{2}$ lbs. of milk.

Mar. 31. Feces fluid and yellowish in color early, becoming less watery later in day. Milk as yesterday.

Apr. 1. Feces normal today. Calf takes 6 lbs. of milk.

Apr. 26. Calf has been normal in condition to date. Now taking 11 lbs. of milk.

Apr. 27. Killed today. No abnormalities found at autopsy.

During this same period of 2 months, twelve calves died of spontaneous scours in the herd from which our animals were taken. Two of these were autopsied.

Calf 904.—Born Mar. 31, of a native cow in her second pregnancy. Calf appeared well on Apr. 1. Diarrhea started Apr. 2. The calf grew weaker and died at noon Apr. 3. The autopsy brought out the following conditions.

Rumen contains a handful of shavings and some opalescent fluid. Similar fluid in fourth stomach totalling about 400 cc. No signs of milk or milk curds in the stomachs. The upper third of the small intestine is markedly congested, similarly the lowest third. Middle third hemorrhagic. The hemorrhages tend to raise the mucous surface into elevated streaks and patches. The large intestine is congested throughout in the form of close longitudinal lines. In the rectum the congestion involves the entire mucosa. Liver cells contain much fat. Sub-endocardial hemorrhages about papillary muscles of left ventricle.

Cultures show large numbers of *B. coli* in spleen, liver, and kidneys.

Calf 934.—Fourth calf of a native cow. Born Apr. 19. Died Apr. 20 at noon. The autopsy showed plenty of colostrum in the fourth stomach. Mucosa a uniform dark red. Small intestine is only moderately congested. There is marked fat stasis indicated by expanded, rather rigid villi full of fat. Urine taken from bladder clear, yellow, contains 0.3 per cent albumin (Esbach).

In this case spleen, liver, and kidneys are sterile. *B. coli* of the usual type isolated from contents of small intestine.

DISCUSSION.

The immediate object of the foregoing experiments was to determine whether colostrum was primarily protective against intestinal bacteria by replacing it by some easily absorbed substance containing the immune bodies found in colostrum. The substance chosen was cow serum.

If the results obtained for and against this assumption had been fairly close they could scarcely be regarded as of any value owing to the number of unknown and largely uncontrollable factors entering into such relatively crude tests. The results point, however, very decidedly in one direction—towards serum as a protective substitute for colostrum. In the first group of five calves receiving serum into a vein and the subcutis, three out of five died and a fourth was very sick. In the second group of five which received the same treatment plus serum fed in the milk none died. In the third group of five which received serum in the milk, two died.

In Table I are brought together certain data bearing on the milk fed and the serum administered. It will be seen that the milk came from three cows. In one case the 1st day's milk came from two other cows. No evidence can be adduced that the milk was responsible for the outcome. The same is true of the serum. The same cow was bled three times and the three lots of serum are designated A, B, and C. The particular lot and its age, counting from the day it was drawn from the cow, are recorded in the table. The age of the serum does not seem to figure in the results obtained. Among the group to which serum was fed, the two that died received the freshest serum of the entire lot.

The calves came from grade stock. Neither breed nor sex nor the number of the pregnancy can be drawn in as having any direct bearing on the outcome.

The results indicate that the cow serum introduced both by way of the blood and the digestive tract replaced colostrum successfully. Simply feeding the serum, though only partially successful, appears superior to injecting it. These figures become more favorable to the

TABLE I.
Calves Treated with Cow Serum in Place of Colostrum.

No.	Breed* and sex.	No. of pregnancy.	Date of birth.	Died. Age.	Killed. Age.	Cow's milk fed.	Age of serum used.	Mode of administration of serum.
				days	days		days	
901	G.; male.	2nd	Mar. 10		34	678 + 712	A-22	Injected.
538	H.; female.	3rd	" 16	1½		678 + 712	" 28	"
485	G.; "	3rd	" 24	3½		678 + 712	" 36	"
767	H.; "	1st	" 24		27	678 + 712	" 36	"
948	" "	1st	May 3	4		712 + 938	B-22	"
905	" male.	4th	Apr. 7		24	678 + 712	A-50	" and fed.
768	" "	2nd	" 10		21	600 + 897	" 53	"
906	" female.	1st	" 11		20	678 + 712	" 54	"
932	" male.	1st	" 17		14	678 + 712	" 60	"
953	G.; female.	4th	May 20		Normal May 30.	712 + 938	C-19	"
772	" male.	1st	Apr. 19	1¾		678 + 712	B-7	Fed.
935	H.; female.	1st	" 21		23	678 + 712	" 9	"
936	" male.	1st	" 21		23	712 + 938	" 9	"
940	" female.	2nd	" 25		19	678 + 712	" 13	"
951	" "	1st	May 8	2½		712 + 938	C-7	"

* G. stands for Guernsey, H. for Holstein.

serum treatment if we take into account certain facts. Though placed with their respective groups, one calf (No. 948) of the first group and one (No. 951) of the third group passed through the test considerably later than the others. With these was a colostrum-fed calf (No. 949) as a control. These all died. Some undetermined factors at this time increased the virulence of scours or reduced the resistance of the three calves, since this control calf is the only one of thirteen colostrum-fed calves to die. The significance of the data will be more impressive if we review the main facts of the preceding article.¹

When colostrum is withheld the body becomes flooded with *Bacillus coli* types. Other forms, such as enterococcus, also appear but they are overgrown in cultures by the more numerous and very vigorous *Bacillus coli* types. After a calf has ingested colostrum or has been treated with serum, the invasion of the body is suppressed. The digestive tract, however, may not have been protected sufficiently to prevent scours or acute diarrhea from appearing after 1 or 2 days. In fatal cases the spleen, liver, and kidneys are shown to be free from bacteria or nearly so. An examination of notes made during the past 5 years on fatal spontaneous scours in colostrum-fed calves shows that with rare exceptions in these cases also there is no *Bacillus coli* invasion of the tissues. In the few animals in which *Bacillus coli* was abundant in such organs as the spleen, liver, and kidneys, the autopsy notes showed that the calf had not suckled the dam at all.

In those cases of fatal scours in which colostrum was found in the fourth stomach there was either incomplete protection of the intestinal tract because the colostrum was ingested late or else the digestive mechanism itself was imperfect.

Among the serum-treated calves which survived and were kept 2 to 8 weeks before being killed, joint lesions were practically absent. Occasional transient lameness was noted which may have been due to temporary localization of bacteria or injury. In two of the calves (Nos. 935 and 936) which received serum in the milk focal interstitial nephritis was found when they were killed at the age of 23 days.

CONCLUSIONS.

The serum of a normal lactating cow when injected into calves a few hours after birth saved only two out of five calves so treated.

Serum added to the milk of the first two meals saved three out of five. When the two methods were combined and the serum was both injected and fed all five calves so treated survived as normal calves. These figures to be significant should be compared with the controls of both series.¹ Since the beginning of this investigation twelve out of thirteen colostrum-fed calves have survived and only four out of fifteen from which colostrum has been withheld.

In those that died the serum whether fed or injected protected the internal organs against the invasion and multiplication of *Bacillus coli* and other intestinal types and in this respect its protective action is equivalent to that of colostrum in those calves which die of spontaneous scours.

THE IODINE CONTENT OF THE BLOOD FOLLOWING THYROIDECTOMY.

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The presence of iodine widely distributed in animal tissues has been well established. The occurrence of iodine in certain essential constituents of the thyroid gland has long been known, and there is evidence that the administration of iodine produces changes in the organ. Little is known concerning the relation of the thyroid to the iodine metabolism of the body.

Baumann¹ first showed that the thyroid gland contains iodine in amounts sufficient for quantitative determination, and obtained evidence that iodine forms an essential constituent of the gland. He found that the thyroids of twenty-six adults from Freiberg contained amounts of iodine varying from 0.22 mg. to 7.2 mg., the average iodine content of these twenty-six glands being 2.5 mg. The thyroids of four others contained 10.8, 14.9, 29.9, and 35.3 mg. respectively. Of thirty-four adults from Hamburg, the thyroids of thirty contained amounts of iodine varying between 0.3 and 9.7 mg. per gland, the average iodine content of the thirty glands being 3.83 mg. The thyroids of the other four individuals contained amounts of iodine in excess of 10 mg. per gland, the greatest amount being 27.6 mg. The thyroids of eleven adults from Berlin had an iodine content of less than 10 mg. per gland, the average content per gland being 6.6 mg. Two other thyroids contained 14.1 and 22.7 mg. respectively. Oswald² has also found a considerable variation in the average iodine content of the thyroid in different localities. Baumann found that the thyroids of children usually contained little or no iodine, and that the presence or absence of iodine and the amount, when present, varied according to the locality from which the glands were obtained. Wells³ analyzed the thyroids of six children under 4 years of age from Chicago; three contained only traces and the other three contained from 0.011 to 0.092 per cent of iodine.

¹ Baumann, E., *Z. physiol. Chem.*, 1895-96, xxi, 319; 1896-97, xxii, 1.

² Oswald, A., *Z. physiol. Chem.*, 1897, xxiii, 265.

³ Wells, H. G., *J. Am. Med. Assn.*, 1897, xxix, 954.

Marine and Williams⁴ and Marine and Lenhart⁵ have arranged a series of thyroid glands from minimum to maximum in accordance with their iodine content and have found that they are at the same time arranged in series according to their histology, their colloid content, and their weight. In other words, the weight of the glands varied directly with the degree of hyperplasia and inversely with the iodine content. Marine and Lenhart⁶ have found that thyroid hyperplasia in fish in any stage is diminished by addition of iodine to the water; they state that hyperplasia may be similarly prevented by iodine.

Loeb⁷ observed hyperplasia in the thyroids of guinea pigs following the removal of part of the gland. He thought that there was evidence of a certain quantitative relation between the degree of hyperplasia and the amount of thyroid removed. Iodine did not diminish the intensity of the hyperplastic changes and he thought indeed that there was some indication that preparations of iodine might increase hyperplasia. Feeding of thyroid preparations, on the contrary, prevented hyperplasia.

Barell⁸ found iodine in the ovaries, adrenal, and spleen of swine and cattle and in various commercial preparations of these organs. Howald⁹ says that under normal conditions iodine is not found in human hair, dog's hair, or feathers of roosters; but following the administration of iodides the hair and feathers contain iodine. There was no iodine in the hair of a cretin 4 years of age, or of a woman with an extensive carcinoma of the thyroid.

Bourcet¹⁰ has studied the distribution of iodine in the tissues of the rabbit. His figures obtained by examination of three rabbits are as follows: 200 gm. of blood contained 0.005 mg. of iodine; 60 gm. of heart, 0.0005 mg.; 400 gm. of liver with bile ducts, 0.71 mg.; 82 mg. of kidney, 0.027 mg.; 400 gm. of fat, none; 50 gm. of hair, 0.9 mg.; 500 gm. of muscle, 0.025 mg.; 40 gm. of lung, 0.03 mg.; 30 gm. of brain, 0.012 mg.; 10 gm. of pancreas, none; 200 gm. of skin without fur, 0.12 mg. Similar results were obtained with the tissues of a dog, iodine being recovered from the thymus, pituitary, and medullary tissue of the brain, mammary gland, and gravid uterus. Bourcet believes that all of the glands of the body contain iodine, though the amount is much less than that which is present in the thyroid. Iodine is excreted through the skin, the hair being the chief agent of elimination. Gley and Bourcet¹¹ find that the blood of dogs contains between 0.013 and 0.06 mg. of iodine per liter, which they think is in combination with protein.

⁴ Marine, D., and Williams, W. W., *Arch. Int. Med.*, 1908, i, 349.

⁵ Marine, D., and Lenhart, C. H., *Arch. Int. Med.*, 1909, iv, 440.

⁶ Marine, D., and Lenhart, C. H., *J. Exp. Med.*, 1911, xiii, 455.

⁷ Loeb, L., *J. Med. Research*, 1919, xl, 199; 1919-20, xli, 485; 1920-21, xlii, 77.

⁸ Barell, E., *Chem. Centr.*, 1897, lxviii, pt. 1, 608.

⁹ Howald, W., *Z. physiol. Chem.*, 1897, xxiii, 209.

¹⁰ Bourcet, P., *Compt. rend. Acad.*, 1900, cxxxi, 392.

¹¹ Gley, E., and Bourcet, P., *Compt. rend. Acad.*, 1900, cxxx, 1721.

Rogoff¹² found in two dogs that blood coming from the hyperplastic thyroid glands which had a very low iodine content did not cause the developmental changes which occur in tadpoles fed with thyroid. Blood of a third dog obtained during massage of the gland or during sympathetic stimulation produced the characteristic developmental changes. The samples of blood from this dog contained less than 0.005 mg. of iodine per gm. of dried blood. Later Rogoff and Goldblatt¹³ used specimens of blood obtained from the glands of fifteen individuals on whom thyroidectomy had been performed. In this group were included instances of exophthalmic goiter, colloid goiter, and adenoma of the thyroid. They obtained no evidence of the presence of the active principle of the thyroid gland when these specimens of blood were fed to tadpoles. Examination of the specimens when sufficient amounts of blood were available failed to show the presence of iodine. Kendall¹⁴ has separated the iodine of the thyroid into two parts. Part A is soluble in alkaline media and produces the same effects as thyroid when administered to patients; Part B is soluble in acids and does not produce the reactions of thyroid when administered to patients. When Marine and Feiss¹⁵ perfused thyroids with solutions of potassium iodide they found that iodine was quickly taken up and fixed and was not readily removed by washing the gland with Ringer's solution. They obtained the same results by injecting the iodine into the circulation. Although, according to Marine and Rogoff,¹⁶ iodine is quickly fixed in the thyroid for a time, it does not alter the influence of the thyroid tissue on the metamorphosis of tadpoles, but after several hours this property is distinctly increased. They regard the fixation of iodine and the formation of metamorphosis-promoting substance as distinct processes and think that the formation of this substance is dependent upon the amount of mother substance present in the gland rather than upon the amount of iodine, provided, however, that iodine is present in the usual quantity.

EXPERIMENTAL.

Observations recorded above concerning the relation of iodine to the thyroid gland have suggested the possibility that the thyroid might modify the iodine content of the blood. A quantitative study of the iodine in the blood has seemed desirable. Kendall¹⁷ has

¹² Rogoff, J. M., *J. Pharmacol. and Exp. Therap.*, 1918-19, xii, 193.

¹³ Rogoff, J. M., and Goldblatt, H., *J. Pharmacol. and Exp. Therap.*, 1921, xvii, 473.

¹⁴ Kendall, E. C., *J. Biol. Chem.*, 1915, xx, 501.

¹⁵ Marine, D., and Feiss, H. O., *J. Pharmacol. and Exp. Therap.*, 1915, vii, 557.

¹⁶ Marine, D., and Rogoff, J. M., *J. Pharmacol. and Exp. Therap.*, 1916, viii, 439; 1916-17, ix, 1.

¹⁷ Kendall, E. C., and Richardson, F. S., *J. Biol. Chem.*, 1920, xliii, 161.

recently published a method for determining iodine in the blood; this method is in reality a modification of his original¹⁸ method of determining iodine in the thyroid gland. With this method, suitable for the determination of very small amounts of iodine, I have first determined the iodine content of the blood of normal dogs kept under laboratory conditions and then removed the thyroids of these animals in order to ascertain whether the iodine content of the blood is changed.¹⁹ Determinations of the iodine in the blood have usually been made in duplicate by Kendall's method; occasionally in order to economize blood single determinations have been made at short intervals. After removal of a thyroid gland the animal has been kept under conditions identical with those before operation. The experiments are described in the order in which they were performed.

Determinations of the iodine in the blood of seventeen dogs have been made before the removal of the thyroid. The lowest amount of iodine found in normal blood is 0.0029 mg. in Experiment 10 and the highest amount is 0.0145 mg. in Experiment 8. The average amount of iodine in the blood of the seventeen dogs before the removal of the thyroid is 0.0079 mg. per 100 cc. of blood. Kendall gives 0.013 mg. of iodine as the average iodine content of the blood. He does not state whether or not the blood was human and does not give its source.

Experiment 1.—No determination of iodine in the blood was made before operation.

June 19, 1921. Both lobes of the thyroid were removed, a small tab being left at each lower pole with the parathyroids.

July 19. 100 cc. of blood contain 0.039 mg. of iodine.

100 cc. of blood contain 0.0383 mg. of iodine.

Oct. 3. 100 cc. of blood contain 0.03 mg. of iodine.

100 cc. of blood contain 0.0283 mg. of iodine.

Oct. 20. Animal killed. The small pieces of thyroid tissue at the lower poles are found to be very much enlarged and quite firm. Microscopic sections show active hyperplasia.

Experiment 2.—

July 16, 1921. 100 cc. of blood contain 0.0116 mg. of iodine.

Aug. 26. 100 cc. of blood contain 0.01 mg. of iodine.

¹⁸ Kendall, E. C., *J. Am. Chem. Soc.*, 1912, xxxiv, 894.

¹⁹ All operations were performed under ether anesthesia.

Oct. 26. 100 cc. of blood contain 0.01 mg. of iodine.

Nov. 1. Both lobes of the thyroid are completely removed, leaving the parathyroid *in situ* at each lower pole.

Nov. 7. 100 cc. of blood contain 0.0141 mg. of iodine.

100 cc. of blood contain 0.014 mg. of iodine.

Nov. 9. The animal is found dead in its cage. At autopsy a large hematoma is found filling the space from which the left thyroid lobe had been removed. There is no suppuration of the wound.

Experiment 3.—

Aug. 25, 1921. 100 cc. of blood contain 0.0083 mg. of iodine.

100 cc. of blood contain 0.0066 mg. of iodine.

Sept. 3. The gland is removed completely, leaving the parathyroids at the lower poles *in situ*.

Sept. 17. 100 cc. of blood contain 0.02 mg. of iodine.

100 cc. of blood contain 0.0208 mg. of iodine.

The animal is very much emaciated and its hair is falling out in considerable quantities. It is killed with ether. The two parathyroids at the lower poles of the thyroid lobe are found intact.

Experiment 4.—

Sept. 15, 1921. 100 cc. of blood contain 0.0041 mg. of iodine.

100 cc. of blood contain 0.005 mg. of iodine.

Oct. 6. Both lobes of the gland are removed with the exception of a small tab at each lower pole.

Oct. 12. 100 cc. of blood contain 0.0166 mg. of iodine.

100 cc. of blood contain 0.0157 mg. of iodine.

Experiment 5.—

Sept. 20, 1921. 100 cc. of blood contain 0.0075 mg. of iodine.

100 cc. of blood contain 0.0083 mg. of iodine.

Oct. 4. Both lobes of the thyroid are completely removed.

Oct. 10. 100 cc. of blood contain 0.025 mg. of iodine.

100 cc. of blood contain 0.0216 mg. of iodine.

The animal is killed with ether. Its operative wound has healed by first intention.

Experiment 6.—

July 21, 1921. 100 cc. of blood contain 0.01 mg. of iodine.

Aug. 1. 100 cc. of blood contain 0.01 mg. of iodine.

Aug. 15. 100 cc. of blood contain 0.01 mg. of iodine.

Sept. 15. 100 cc. of blood contain 0.01 mg. of iodine.

100 cc. of blood contain 0.00833 mg. of iodine.

Oct. 21. Both lobes of the gland are removed, leaving the parathyroids at the lower poles *in situ*.

Oct. 30. 100 cc. of blood contain 0.03 mg. of iodine.

100 cc. of blood contain 0.0316 mg. of iodine.

The animal is killed with ether. The wound has healed by first intention.

Experiment 7.—

Oct. 19, 1921. 100 cc. of blood contain 0.0125 mg. of iodine.

100 cc. of blood contain 0.0133 mg. of iodine.

Nov. 8. Both lobes of the thyroid are removed, leaving the parathyroid at the lower pole *in situ*.

Nov. 13. 100 cc. of blood contain 0.025 mg. of iodine.

100 cc. of blood contain 0.0266 mg. of iodine.

Nov. 15. The animal is found dead in its cage. There was no clonic convulsion of tetany before death. There is slight suppuration of the wound at autopsy.

Experiment 8.—

Nov. 15, 1921. 100 cc. of blood contain 0.015 mg. of iodine.

100 cc. of blood contain 0.0141 mg. of iodine.

Dec. 1. The usual operation for removal of the thyroid is performed, leaving the parathyroid at each lower pole.

Dec. 3. 100 cc. of blood contain 0.0166 mg. of iodine.

Dec. 5. 100 cc. of blood contain 0.015 mg. of iodine.

There is slight suppuration of the wound.

Dec. 7. 100 cc. of blood contain 0.0066 mg. of iodine.

There is considerable discharge from the wound and the animal does not eat.

Dec. 9. 100 cc. of blood contain 0.0066 mg. of iodine.

The animal is killed with ether because its wound is suppurating and it does not eat.

Experiment 9.—

Dec. 3, 1921. 100 cc. of blood contain 0.00833 mg. of iodine.

100 cc. of blood contain 0.0075 mg. of iodine.

Dec. 3. The usual operation for removal of both lobes of the thyroid is performed, leaving a small tab of thyroid tissue with each parathyroid at the lower pole.

Dec. 5. 100 cc. of blood contain 0.02832 mg. of iodine.

Dec. 7. 100 cc. of blood contain 0.0375 mg. of iodine.

Dec. 8. The animal is found dead in its cage, symptoms of tetany having been present during 24 hours. Autopsy shows a small tab of thyroid tissue at each lower pole. These tabs of tissues are deep red in color. The parathyroids are not identified.

Experiment 10.—

Dec. 10, 1921. 100 cc. of blood contain 0.0033 mg. of iodine.

100 cc. of blood contain 0.0025 mg. of iodine.

Dec. 12. The usual operation for removal of both lobes is performed, leaving a small tab of thyroid tissue at each lower pole with the parathyroids.

Dec. 14. 100 cc. of blood contain 0.01 mg. of iodine.

Dec. 18. 100 cc. of blood contain 0.0116 mg. of iodine.

Following the operation this animal has eaten little. Immediately after removal of blood there are clonic convulsions of tetany and death occurred on Dec. 21.

At autopsy the wound is closed and there is no suppuration but the deeper tissues about the remaining bits of thyroid are red and indurated.

Experiment 11.—

Dec. 13, 1921. 100 cc. of blood contain 0.01 mg. of iodine.

100 cc. of blood contain 0.01 mg. of iodine.

Jan. 30, 1922. 100 cc. of blood contain 0.00833 mg. of iodine.

Feb. 1. The thyroid is removed as usual, leaving a small tab of thyroid tissue at each lower pole.

Feb. 6. The wound has healed by primary intention but there has been somewhat more edema than usual for several days. The animal has had today clonic convulsions of tetany. It is given 15 cc. of a 30 per cent solution of calcium lactate intraperitoneally and convulsions have disappeared within $\frac{1}{2}$ hour.

Feb. 8. The calcium lactate is repeated because of clonic convulsions of tetany.

Feb. 11. 100 cc. of blood contain 0.015 mg. of iodine.

100 cc. of blood contain 0.02 mg. of iodine.

The animal has lost much weight and its hair is falling out in considerable quantities. Eats little.

Feb. 16. The animal is found dead in its cage. No suppuration of the wound is observed at autopsy. The bits of thyroid tissue at each lower pole are embedded in scar tissue.

Experiment 12.—

Dec. 18, 1921. 100 cc. of blood contain 0.0066 mg. of iodine.

100 cc. of blood contain 0.0066 mg. of iodine.

Dec. 23. The thyroid is removed, leaving the parathyroids at each lower pole.

Dec. 27. 100 cc. of blood contain 0.011 mg. of iodine.

Dec. 29. 100 cc. of blood contain 0.0097 mg. of iodine.

Dec. 31. 100 cc. of blood contain 0.00833 mg. of iodine.

The animal died following the removal of blood. At autopsy no suppuration of wound is found.

Experiment 13.—

Oct. 28, 1921. 100 cc. of blood contain 0.005 mg. of iodine.

100 cc. of blood contain 0.00583 mg. of iodine.

Jan. 10, 1922. The thyroid gland is removed.

Jan. 14. 100 cc. of blood contain 0.0233 mg. of iodine.

100 cc. of blood contain 0.02 mg. of iodine.

Jan. 15. The animal is found dead in its cage. No suppuration of the wound is found at autopsy.

Experiment 14.—

Jan. 3, 1922. 100 cc. of blood contain 0.0055 mg. of iodine.

100 cc. of blood contain 0.0055 mg. of iodine.

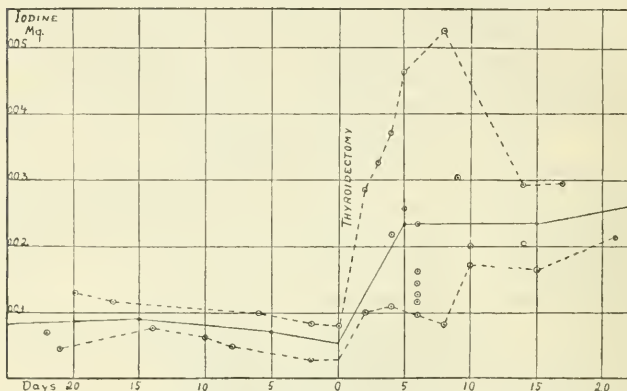
Jan. 11. The thyroid is removed.

Jan. 14. 100 cc. of blood contain 0.0333 mg. of iodine.

100 cc. of blood contain 0.036 mg. of iodine.

The animal died while being bled. No suppuration of the wound is found at autopsy.

This group of experiments demonstrates that the amount of iodine in the blood is greatly increased after thyroidectomy (Text-fig. 1). This increase is seen in all experiments. In Experiment 8 the increase is small and the amount of iodine in the blood soon falls below that found before operation; but this dog has suffered from a badly infected wound and did not eat, so that its store of iodine has not been re-



TEXT-FIG. 1. Composite chart of iodine determinations made before and after thyroidectomy. Maximum and minimum determinations are connected by broken lines to show the degree of variation. Only one determination was made on the day of operation, since it seemed undesirable to draw blood at this time. The date of operation has been crossed by the minimum line at the level of the last determination. The continuous line is an average of all determinations made within a period of 10 days, the date of operation being crossed midway between the minimum and maximum lines.

plenished. Increase of iodine in the blood varies but in a number of experiments is considerable. For example, in Experiment 14 there is an increase after removal of the thyroid from 0.0055 mg. to 0.033 mg. in 100 cc. of blood, and in Experiment 13 the increase is from 0.00583 mg. of iodine before operation to 0.0233 mg. per 100 cc. of blood after thyroidectomy. These variations are too great to be explained by experimental error, for Kendall has shown that there is a constant

loss of about 3 per cent of iodine determined by his method. The change noted after operation cannot be due to difference in the iodine content of the food, for in a number of instances repeated determinations before operation when the food was identical with that received after operation show little variation. For example, Experiment 6 shows a variation from 0.01 mg. to 0.00833 mg. among iodine determinations made on four occasions between July 21 and September 15; during this time the dog received the same diet as after the operation so that an increase from 0.01 mg. to 0.03 or 0.0316 mg. of iodine per 100 cc. of blood within the first 10 days after operation cannot be explained by variation of the iodine in the food.

It is noteworthy that the increase of iodine occurs soon after removal of the thyroid gland; in Experiment 9 there is an increase from 0.00833 mg. to 0.02832 mg. of iodine per 100 cc. of blood, and in Experiment 10 from 0.0033 mg. to 0.01 mg. within the first 48 hours after removal of the thyroid gland. If this sudden increase in the iodine content of the blood were due to injury of the gland during the operation the iodine content of the blood would return to normal in a very short time, but in Experiment 5, 6 days after operation the iodine content of the blood is 0.025 mg. per 100 cc. of blood, or three times that before operation, and in Experiment 3, 14 days after operation it is 0.0208 mg. as compared with 0.0083 mg. before operation; in Experiment 1, 30 days after operation the iodine content of the blood is 0.0386 mg. and 106 days after operation it is 0.0291 mg. per 100 cc. of blood.

The possibility suggests itself that the thyroid influences the metabolism of iodine in much the same way that the pancreas controls the metabolism of glucose. Present knowledge of the subject does not establish any close analogy between iodine and carbohydrate metabolism.

A series of experiments has been performed to determine the effect of thyroid feeding on the iodine content of the blood of dogs of which the thyroids had been removed. Following the operation determinations of the iodine content of the blood have been made and the dogs have then been fed with fresh thyroid of sheep in addition to the usual food. After a number of days, the iodine content of the blood has again been determined. The thyroid feeding has been discontinued and later iodine determinations have been made.

Experiment 15.—

July 15, 1921. 100 cc. of blood contain 0.01 mg. of iodine.

Aug. 25. 100 cc. of blood contain 0.0125 mg. of iodine.

Oct. 15. 100 cc. of blood contain 0.0116 mg. of iodine.

Nov. 1. Both lobes of the gland are removed, the parathyroids at the lower pole being identified and left *in situ*.

Nov. 11. The wound has healed by first intention.

100 cc. of blood contain 0.02 mg. of iodine.

100 cc. of blood contain 0.02 mg. of iodine.

Nov. 22. 100 cc. of blood contain 0.0233 mg. of iodine.

100 cc. of blood contain 0.0233 mg. of iodine.

Jan. 5, 1922. 100 cc. of blood contain 0.021 mg. of iodine.

100 cc. of blood contain 0.021 mg. of iodine.

Following the removal of blood the feeding of fresh thyroids of sheep was started. The dog received from two to four lobes each day.

Jan. 19. 100 cc. of blood contain 0.01 mg. of iodine.

100 cc. of blood contain 0.012 mg. of iodine.

Experiment 16.—

Dec. 13, 1921. 100 cc. of blood contain 0.0066 mg. of iodine.

100 cc. of blood contain 0.0066 mg. of iodine.

Dec. 20. The thyroid is removed, leaving the parathyroid at each lower pole.

Dec. 25. 100 cc. of blood contain 0.0483 mg. of iodine.

Dec. 28. 100 cc. of blood contain 0.0566 mg. of iodine.

Jan. 3, 1922. 100 cc. of blood contain 0.0333 mg. of iodine.

Jan. 6. 100 cc. of blood contain 0.0333 mg. of iodine.

The feeding of fresh thyroid of sheep was started. The animal receives from five to eight lobes each day.

Jan. 19. 100 cc. of blood contain 0.0066 mg. of iodine.

100 cc. of blood contain 0.0075 mg. of iodine.

Jan. 20. The thyroid feeding is discontinued.

Feb. 14. 100 cc. of blood contain 0.0216 mg. of iodine.

100 cc. of blood contain 0.0233 mg. of iodine.

The animal is killed with ether following the removal of blood.

Experiment 17.—

Dec. 29, 1921. 100 cc. of blood contain 0.007 mg. of iodine.

100 cc. of blood contain 0.007 mg. of iodine.

Jan. 20, 1922. The thyroid is removed, leaving a very small tab of thyroid tissue at each lower pole with the parathyroid.

Jan. 27. The animal is losing its hair in considerable quantities.

The feces are very hard, dry, and powdery. The wound has healed by first intention.

Feb. 4. 100 cc. of blood contain 0.0166 mg. of iodine.

100 cc. of blood contain 0.0168 mg. of iodine.

Feb. 9. The feeding of fresh thyroid of sheep is started, the animal receiving from six to eight lobes each day.

Feb. 15. 100 cc. of blood contain 0.0066 mg. of iodine.
The hair has stopped falling out and the animal has somewhat softer feces.

Mar. 1. The feeding of fresh thyroid is discontinued.

Mar. 20. The hair is falling out in considerable quantities, and the feces are hard and powdery.

100 cc. of blood contain 0.015 mg. of iodine.

Experiment 18.—

Feb. 1, 1922. 100 cc. of blood contain 0.00583 mg. of iodine.

100 cc. of blood contain 0.0066 mg. of iodine.

Feb. 11. The thyroid is completely removed, leaving only the parathyroids at each lower pole.

Feb. 17. 100 cc. of blood contain 0.0133 mg. of iodine.

100 cc. of blood contain 0.0116 mg. of iodine.

The hair is falling out in considerable quantities and the animal is losing weight. There are mild clonic convulsions of tetany.

Feb. 19. The feeding of fresh thyroid is started, the animal receiving from four to eight lobes each day.

Feb. 23. The hair has stopped falling out and the animal is gaining in weight. There are no symptoms of tetany.

100 cc. of blood contain 0.008 mg. of iodine.

The thyroid feeding is discontinued.

Mar. 5. The animal is losing weight, eats very little, and the hair is falling out.

100 cc. of blood contain 0.0125 mg. of iodine.

The animal is killed with ether. The parathyroids are found embedded in scar tissue at each lower pole.

In this group of experiments the foregoing results are confirmed and further evidence that increase of the iodine content of the blood is due to the removal of the thyroid is obtained. Feeding of fresh thyroid of sheep to thyroidectomized dogs reduces the iodine content of the blood so that it approximates the preoperative level. In Experiment 15 the iodine content of the blood before operation was 0.0125 mg. per 100 cc., and on the day the animal first received fresh thyroid with its food the postoperative iodine of the blood was 0.021 mg.; after 14 days of thyroid feeding the iodine content of the blood had fallen to 0.012 mg.

In Experiment 16 the preoperative iodine content of the blood was 0.0066 mg. with a postoperative iodine content which reached the height of 0.0566 mg. and was still 0.033 mg. after the removal of 400 cc. of blood within a period of 12 days. 13 days after thyroid feeding

had been started the iodine content of the blood was 0.0075 mg. In Experiments 17 and 18 the iodine content of the blood rose again to the former postoperative level when the thyroid feeding was discontinued.

When some substance which the thyroid gland normally supplies is lost as the result of removal of the organ, the iodine content of the blood increases to an abnormal level. This hypothetical substance may be restored by feeding thyroid, and under this influence the normal iodine level is restored. Iodine in the blood decreases even though the thyroid gland is no longer present to remove it from the blood. The thyroid supplies some substance which profoundly influences the metabolism of iodine not only within the thyroid but elsewhere in the body.

CONCLUSIONS.

1. After thyroidectomy in dogs the iodine content of the blood is increased.

2. The administration of fresh thyroid gland of sheep by mouth to thyroidectomized dogs causes the iodine content of the blood to fall so that it returns to the normal preoperative level; when the thyroid feeding is discontinued the iodine content of the blood is again increased.

It is with pleasure that I acknowledge the advice and encouragement given by Professor E. L. Opie.

BLOOD DESTRUCTION DURING EXERCISE.

I. BLOOD CHANGES OCCURRING IN THE COURSE OF A SINGLE DAY OF EXERCISE.

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The studies of Rous and Robertson¹ have shown that normally the destruction of erythrocytes is accomplished, in part at least, by a process of fragmentation in the blood stream. There is evidence that the breaking up is the result of mechanical injury. Among physiologic conditions which would favor such disintegration, vigorous exercise at once suggests itself. Both the increased rapidity of blood flow and the violent muscular contractions would tend to increase the wear and tear upon the corpuscles. The present investigations have been undertaken with the object of determining whether an increased rate of blood destruction during exercise could be demonstrated.

Since the subject is closely linked with the general question of fluid and cell changes during exercise, a number of observations have been made in this connection, and it is with these that the present paper will be largely concerned. The data given serve as controls to the method employed in experiments subsequently to be reported.

The changes in the blood during exercise are not yet completely understood. The early literature upon the subject has been well summarized by Hawk.² Practically without exception, investigators have reported an increase in hemoglobin and corpuscles immediately after short periods of exercise. Schneider and Havens³ confirmed these findings in observations made at low altitudes, but following exercise at high altitudes found no increase in either cells or pigment. On the

¹ Rous, P., and Robertson, O. H., *J. Exp. Med.*, 1917, xxv, 651.

² Hawk, P. B., *Am. J. Physiol.*, 1903-04, x, 384.

³ Schneider, E. C., and Havens, L. C., *Am. J. Physiol.*, 1914-15, xxxvi, 380.

other hand, Cohnheim and his coworkers⁴ as well as Gross and Kestner⁵ have shown an actual decrease in red count and per cent of hemoglobin after long and strenuous mountain climbing. This they attribute to dilution of the blood with fluid derived from the muscles. The workers who have found a concentration of the blood during exercise explain it either by a changed distribution of the corpuscles in the vessels² or by loss of fluid into the muscles⁶ or liver lymphatics.⁷

Obviously the points at issue can only be cleared up by accurate determinations of cell and plasma volume during exercise. So far as we are aware, no such observations have been reported. The lack of a method of estimating blood volume which is at once accurate and innocuous has no doubt hitherto prevented advancement along this line.

Method.

Two types of blood volume determination have shown themselves to be of practical value for use on the living animal. The first is the well known carbon monoxide inhalation method, originally devised by Gréhan and Quinquaud.⁸ The second entails the addition of some substance to the blood plasma and the subsequent determination of its degree of dilution. Perhaps the most satisfactory of the numerous substances employed in this way is the dye, vital red, first used by Keith, Rowntree, and Geraghty.⁹ The non-toxic nature of the dye allows repetition of the volume determinations at short intervals. Since the carbon monoxide method cannot readily be repeated within a single day, the dye method was adopted for use in the present series of experiments.

The technique followed closely that described in detail by Hooper, Smith, Belt, and Whipple¹⁰ and Smith.¹¹ Mention need be made only of slight modifications.

⁴ Cohnheim, O., Kreglinger, and Kreglinger, *Z. physiol. Chem.*, 1909, lxxiii, 413.

⁵ Gross, W., and Kestner, O., *Z. Biol.*, 1920, lxx, 187.

⁶ von Willebrand, E. A., *Skandin. Arch. Physiol.*, 1903, xiv, 176.

⁷ Lamson, P. D., *J. Pharmacol. and Exp. Therap.*, 1920-21, xvi, 125.

⁸ Gréhan and Quinquaud, E., *Compt. rend. Acad.*, 1882, xciv, 1450.

⁹ Keith, N. M., Rowntree, L. G., and Geraghty, J. T., *Arch. Int. Med.*, 1915, xvi, 547.

¹⁰ Hooper, C. W., Smith, H. P., Belt, A. E., and Whipple, G. H., *Am. J. Physiol.*, 1920, li, 205.

¹¹ Smith, H. P., *Am. J. Physiol.*, 1920, li, 221.

Vital red was used in 1 per cent strength for injection, and dogs of 10 to 15 kilo body weight received 4 cc. of it, while those above 15 kilos received 5 cc. In making the dilute dye solution for the preparation of a standard, 1 cc. of the 1 per cent dye solution was made up with distilled water to 250 cc. in a volumetric flask. This gave a solution of the strength of $1/25,000$, a figure easier to deal with than the fraction $1/26,666.67$ employed in the articles referred to above. It was found to be quite important to allow the blood to resume its normal flow for several minutes after the manipulations necessary to the introduction into the vein of the aspirating needle, since stasis caused by pressure on the vessel changes the hematocrit value. The procedure employed was as follows:

The needle, firmly grasped at the base by a long jawed hemostat, is connected to a syringe containing a little saline solution. While an assistant makes pressure upon the external jugular vein just above the clavicle, the needle is thrust into its lumen. The aspiration of blood into the saline solution shows clearly when the needle is in place. Pressure on the vein is then discontinued, the syringe is detached, leaving the needle in place, and a close fitting stylet is introduced into the lumen of the latter, completely closing it. After 2 or 3 minutes have been permitted to elapse, during which presumably the normal flow in the vein is completely reestablished, the stylet is withdrawn and a sample of blood taken into a syringe. The same method is used in obtaining both specimens required for each volume determination. In the case of the second specimen it has the special advantage of insuring the accuracy of the 4 minute period between the dye injection and the withdrawal of the blood.

The hematocrit determinations were made by filling three tubes of the type described by Epstein¹² from each specimen of blood and centrifuging these at 3,500 revolutions per minute for 30 minutes. The average plasma per cent of the three tubes, after correction for oxalate dilution, was taken as the true plasma per cent of the blood. To obtain the plasma for colorimetric readings, the centrifuge tubes were centrifuged at 2,000 revolutions per minute for 10 to 15 minutes. This modification, while adding somewhat to the complexity of the method, has given more uniform results than the use of graduated centrifuge tubes.

Hemoglobin determinations were made by the method of Newcomer.¹³ The colored glass standard had been checked against gasometrically standardized Palmer¹⁴ and Robscheit¹⁵ solutions and found to correspond closely to these throughout a wide range. The specimen for hemoglobin was withdrawn from the oxalated blood of the jugular vein as obtained for the volume determinations. The reading was subsequently corrected for dilution of blood with oxalate. Per cent of hemoglobin is given in terms of the Haldane scale. 100 per cent hemoglobin on this

¹² Epstein, A. A., *J. Lab. and Clin. Med.*, 1915-16, i, 610.

¹³ Newcomer, H. S., *J. Biol. Chem.*, 1919, xxxvii, 465.

¹⁴ Palmer, W. W., *J. Biol. Chem.*, 1918, xxxiii, 119.

¹⁵ Robscheit, F. S., *J. Biol. Chem.*, 1920, xli, 209.

scale is equivalent to an oxygen-carrying capacity of 18.5 cc. per 100 cc. of whole blood. This corresponds to 13.8 gm. of hemoglobin per 100 cc. of whole blood.

The few red counts recorded were made in duplicate from the oxalated blood of the jugular vein, subsequently corrected for oxalate dilution. Hayem's fluid was used and the Bürker-Zeiss counting chamber.

In reporting the results the term "pigment volume" has been used in the sense in which Whipple and his coworkers¹⁶ employed it; namely, as the product of the blood volume by the per cent of hemoglobin. Subject to the obvious limitations of the vital red method, it will serve as an index of the total circulating blood pigment. But since the hematocrit and hemoglobin values of blood specimens withdrawn from the jugular vein are influenced by changes in cell distribution, this factor must be borne in mind in interpreting the results.

Experimental Procedure.

The chief reason for choosing dogs as the experimental animals, apart from their general utility, was the special susceptibility of their red corpuscles to mechanical injury.¹⁷ It seemed probable that because of this characteristic the blood destruction during exercise would be greater than in the other available species.

The animals were kept in individual cages and fed upon a mixed diet containing considerable meat. To avoid possible changes in blood volume resulting from the ingestion of food, food was not given upon the day of the experiment until after the last blood volume had been completed. But it was found necessary to allow water freely.

For purposes of exercise, treadmills were used, so constructed that the tread was at an angle of 20° to the horizontal. A bicycle cyclometer attached to the machine recorded the number of miles traveled. The standard day's exercise consisted of two periods, each of 2 hours, separated by a rest of 1 hour. The distances covered during these periods varied considerably, being chiefly limited by the ability of the animals to withstand the exertion. All of the animals worked actively, yet the average per day for the series was only 3.8 miles. This may

¹⁶ Whipple, G. H., Hooper, C. W., and Robschey, F. S., *Am. J. Physiol.*, 1920, liii, 151.

¹⁷ Rous, P., and Turner, J. R., *J. Exp. Med.*, 1916, xxiii, 219.

seem surprisingly low, but the fact should be recognized that the 20° inclination of the tread necessitated a vertical ascent of 6,870 feet in this distance. A careful oversight was maintained to prevent exhaustion. Except in the initial period of 10 to 15 minutes exercise, no attempt was made to insure a continuous run, brief rest being allowed as needed. However, immediately before the last of the three blood examinations, the animal was run continuously for about 10 minutes.

It should be noted that the twelve complete experiments here reported were all done during warm weather and panting was regularly induced.

Two series of experiments were performed which will be designated respectively the "exercise series" and the "control series."

Three animals, Nos. 3, 4, and 7, appear in both series of experiments, and in the exercise series Nos. 3 and 5 appear twice. The repetition of experiments on the same animal was in most cases carried out only after the lapse of a considerable time, and during the interval some change in body weight occasionally occurred. The experiments on No. 8 and the first of the two on No. 5 were made when the animals had not completely recovered from an anemia induced by an hemolytic serum. The conditions in these cases were not entirely normal, yet the blood changes noted are of the same general character as those of the other animals of the series. We have, however, deemed it advisable to list the results with them separately in Table I, which shows the "normal" blood findings of the animals. All of the other animals were in good health at the time of observation.

The exercise experiments were conducted in the following manner. The dog was removed directly from cage to table and the first blood volume specimens were taken. From 15 to 20 minutes later the animal was placed on the treadmill and exercised vigorously for 10 to 15 minutes, then quickly removed, and a second set of determinations on the blood made at once. Following this the animal was exercised for 2 hours, allowed to rest for 1 hour, and again exercised for another 2 hour period. Finally, at the end of this time, the third blood volume determination was made. This procedure was slightly altered in the case of Dogs 1 and 7 which were exercised for 3 consecutive hours between the second and third blood volume determinations, without rest or access to water. In the first experiment on Animals 3 and 5, and in the single experiment on Animals 6 and 8, the blood was examined before exercise and after 10 minutes exercise. A third examination was not made.

In the control series the three blood examinations were made at approximately the same intervals as in the exercise series. In the case of Dogs 10 and 3, only two determinations were carried out, an interval of 30 minutes separating those of Dog 10 and 6 hours those of Dog 3.

TABLE I.
Blood Findings at Initial Examination.

Series.	Animal No.	Body weight.	Plasma volume.	Plasma per 100 gm. of body weight.	Cell volume.	Cells per 100 gm. of body weight.	Entire blood volume.	Blood volume per 100 gm. of body weight.	Hemo-globin per 100 gm. of body weight.	Remarks.
		kg.	cc.	cc.	cc.	cc.	cc.	cc.	gm.	
Exercise series.	{ 1 2 3 3 4 5 6 7 }	20½	1,078	5.25	782	3.82	1,860	9.08	1.39	6 days after control observation noted below.
		14½	923	6.36	717	4.95	1,640	11.30	1.85	
		20¼	1,244	6.14	936	4.62	2,180	10.75		
		18¼	1,190	6.53	968	5.30	2,158	10.62	1.93	3 mos. later still.
		18¼	1,226	6.54	818	4.36	2,044	10.90	1.70	3 " before control observations noted below.
		14	940	6.71	408	2.91	1,348	9.64	1.18	2 mos. after observations noted below.
		20¼	983	4.74	967	4.66	1,950	9.40		3 mos. before control observations noted below.
18½	1,030	5.58	812	4.39	1,842	9.96	1.54			
Control series.	{ 9 10 11 12 3 4 7 }	11½	703	5.98	507	4.31	1,210	10.30	1.68	6 days before first exercise observation.
		14¼	719	5.04	723	5.07	1,442	10.11	1.77	
		14½	736	4.99	563	3.82	1,299	8.80	1.35	
		11½	692	5.89	610	5.19	1,302	11.10	2.08	
		20¼	1,174	5.80	894	4.41	2,068	10.20		
		20¼	1,402	6.77	683	3.29	2,058	9.91	1.20	3 mos. after exercise observation.
		16¼	998	6.13	723	4.45	1,721	10.60	1.67	3 " " "
Average.....				5.89		4.37		10.18		1.61

We have purposely omitted the following blood volume determinations of Animals 5 and 8 from the normal series as the animals had not entirely recovered from anemia due to the injection of an hemolytic serum. Their reactions to exercise did not differ from those of normal animals.

5	14	941	6.72	328	2.34	1,269	9.06	2 mos. previous to observation noted above.
8	10½	849	8.29	405	3.95	1,254	12.24	

The Blood in the Resting Animal.

The findings in the initial blood specimen in both series of animals are given in Table I. This specimen is assumed to yield the normal relation of the blood components during rest. The figures obtained compare closely with those reported for normal dogs by Smith, Arnold, and Whipple.¹⁸ The average cell volume is somewhat smaller and the plasma volume slightly larger than they found by the use of the dye method. The cell volume figures, however, correspond almost exactly to their determinations made by the carbon monoxide method, which they regard as yielding figures that closely approximate the true cell volume. The accompanying high hemoglobin content of the blood shows that the differences noted are not due to anemia.

The Effect of Brief Exercise.

The changes which occurred during exercise are recorded in Table II and are graphically shown in Text-fig. 1. Since the first examination of the blood is taken to indicate the normal condition in each animal while resting, there is shown in the table to the right of the actual figure for each determination its percentage in terms of the findings at the first determination. In Text-fig. 1 the percentage changes in each of the blood components are shown and compared with the initial finding.

It will be noted that 10 to 15 minutes of active exercise caused usually a slight increase in plasma volume and quite regularly a marked increase in cell volume, hemoglobin, pigment volume, and number of corpuscles per cubic millimeter. For the series there is an average increase in plasma of 3.6 per cent. In two individuals it showed a slight decrease. The cell volume increased in every case, averaging 12.3 per cent, with a variation from 1.5 per cent to 24.0 per cent. Pigment volume follows closely the changes in cell volume, the increase ranging from 5 to 25 per cent with an average of 16.1 per cent. The per cent of hemoglobin shows a maximal rise of 13 and a minimal of 2, the average being 8.5. The increase in number of red corpuscles per cubic millimeter averages 11 per cent for the two instances followed.

¹⁸ Smith, H. P., Arnold, H. R., and Whipple, G. H., *Am. J. Physiol.*, 1921 lvi, 336.

The Changes Consequent on More Prolonged Exercise.

After several hours of exercise, the plasma volume shows consistently an increase. The average rise above the resting normal is 11.7 per cent, with minimal and maximal variations of 6.8 and 18 per cent. The average increase over the plasma volume after 10 minutes of exercise is 8.1 per cent. In one instance there is a decrease in plasma volume, but of less than 1 per cent, between the second and third determinations. The greatest increase observed is 13 per cent. On the other hand, cell volume, while remaining at an average of 9.4 above the resting cell volume, shows an average decrease of 2.9 per cent below the average found at the end of 10 minutes exercise. In two instances there are increases of 1 and 2.5 per cent. The tendency of the hemoglobin percentage to decrease is more marked but this is, in part at least, due to dilution of the blood by increase in plasma volume. However, the calculated pigment volume, in which this error of dilution is corrected, shows after the period of prolonged exercise a 7.7 per cent fall below the average after 10 minutes of exercise. In no instance is an increase to be found. The final average pigment volume is, however, 8.4 per cent above the average of the first determination. The red count in the two instances followed shows a marked decrease after the prolonged exercise, part of which may be ascribed to the dilution of the blood referred to above.

The Control Series.

Although the animals used as controls were kept in small cages throughout the periods of observation, it was not possible to prevent a certain amount of activity on their part. The dogs varied much in this respect. It is not surprising, then, that the results obtained vary somewhat too (see Table III and Text-fig. 2). Nevertheless they are in striking contrast with those of the exercise series (compare Tables II and III). There is an average increase of 4.6 per cent in the second plasma volume over the first, an increase which closely corresponds to that observed in animals exercised for 10 minutes. It is quite possible that in both series a portion of this increase may be due to the disturbing influence of the first blood volume determination, in which approximately 20 cc. of blood is withdrawn and 4 to 5 cc.

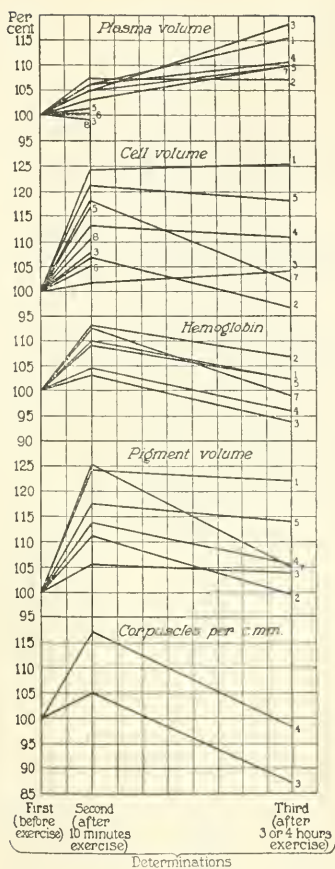
TABLE II.
The Changes during Exercise.

Animal.	Elapsed time.	Circumstances.	Plasma volume.		Cell volume.		Hemoglobin.		Pigment volume.		Red corpuscles.	
			C.	In percentage of the first plasma volume here given.	C.	In percentage of the first cell volume here given.	Per cent.	In percentage of the first hemoglobin determination here given.	Units.	In percentage of the first pigment volume here given.	Per c. min.	In percentage of the first red count here given.
1. Female collie; weight 20½ kilos.	0 30 min.	Before exercise. After 10 min. exercise.	1,078	100.0	782	100.0	111.0	100.0	2,062	100.0		
			1,140	106.0	970	124.0	122.0	110.0	2,570	124.0		
	3 hrs., 30 min.	After 3 hrs. exercise. Had run 3 miles. No rest period.	1,242	115.0	979	125.0	113.0	102.0	2,512	122.0		
2. Male mongrel; weight 14½ kilos.	0 30 min.	Before exercise. After 10 min. exercise.	923	100.0	717	100.0	119.0	100.0	1,950	100.0		
			992	107.5	764	106.5	123.0	113.0	2,160	111.0		
	3 hrs., 30 min.	After 4 hrs. exercise. Had run 4 miles in all with an interpolated hr. of rest.	985	106.8	691	96.5	116.0	106.5	1,944	99.8		

[illegible]

TABLE II—*Concluded.*

Animal.	Elapsed time.	Circumstances.	Plasma volume.		Cell volume.		Hemoglobin.		Pigment volume.		Red corpuscles.	
			Cc.	In percentage of the first given.	Cc.	In percentage of the first cell volume here given.	Per cent.	In percentage of the first hemoglobin determination here given.	Units.	In percentage of the first pigment volume here given.	Per c. mm.	In percentage of the first red count here given.
6. Male setter; weight 20½ kilos.	0 30 min.	Before exercise. After 10 min. exercise.	983	100.0	967	100.0						
			990	100.5	1,025	105.0						
7. Male mongrel; weight 18½ kilos.	0 30 min.	Before exercise. After 10 min. exercise. No rest period.	1,030	100.0	812	100.0	112.0	100.0	2,062	100.0		
			1,080	105.0	960	118.0	126.0	112.5	2,570	125.0		
		After 3 hrs. exercise. Had run 3 miles.	1,132	110.0	830	102.0	110.0	98.5	2,160	105.0		
8. Male bull mongrel; weight 10½ kilos.	0 4 hrs.	Before exercise. After 10 min. exercise.	849	100.0	405	100.0						
			845	99.5	447	110.5						

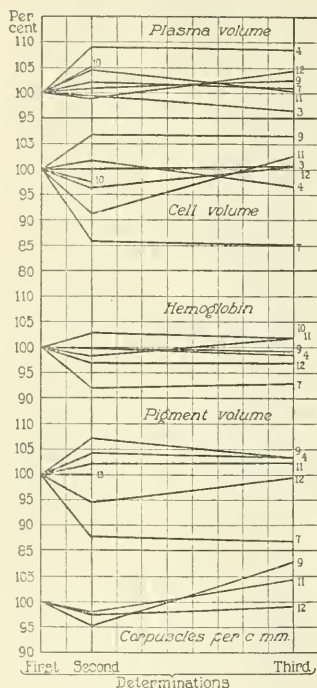


TEXT-FIG. 1. Percentage changes in the blood components during exercise.

TABLE III.
Control Findings.

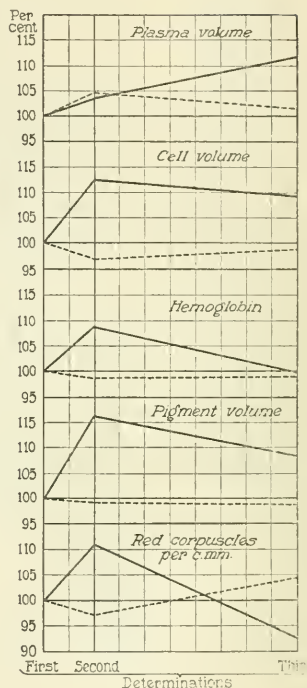
Animal.	Elapsed time.	Circumstances.	Plasma volume.		Cell volume.		Hemoglobin.		Pigment volume.		Red corpuscles.	
			Cc.	In percentage of the first given.	Cc.	In percentage of the first cell volume here given.	Per cent.	In percentage of the first hemoglobin determination here given.	Units.	In percentage of the first pigment volume here	Per c. mm.	In percentage of the first red count here given.
9. Male mongrel; weight $11\frac{3}{4}$ kilos.	0	No exercise on this day.	703	100.0	507	100.0	118.0	100.0	1,437	100.0	8,000,000	100.0
	30 min.		708	100.8	544	107.0	119.0	100.9	1,490	104.0	7,600,000	95.0
	5 hrs., 30 min.		717	102.0	543	107.0	117.5	99.6	1,480	103.0	8,600,000	107.2
10. Male mongrel; weight $14\frac{1}{4}$ kilos.	0	No exercise on this day. Only two blood volume determinations made.	719	100.0	723	100.0	127.0	100.0	1,832	100.0		
	30 min.		752	105.0	716	97.7	125.0	98.5	1,832	100.0		
	5 hrs., 30 min.						130.0	102.0				
11. Male mongrel; weight $14\frac{3}{4}$ kilos.	0	No exercise on this day.	736	100.0	563	100.0	111.0	100.0	1,440	100.0	7,800,000	100.0
	30 min.		770	104.5	514	91.5	114.0	103.0	1,465	102.0	7,600,000	97.5
	5 hrs., 30 min.		730	99.8	581	103.0	112.0	102.0	1,490	102.0	8,100,000	103.8

12. Male mongrel; weight $11\frac{3}{4}$ kilos.	0	No exercise on this day.	692	100.0	610	100.0	134.0	100.0	1,748	100.0	8,200,000	100.0
	30 min, 5 hrs., 30 min.		682 724	99.0 104.0	588 614	96.5 101.0	130.0 130.0	97.1 97.1	1,652 1,740	94.5 99.5	8,000,000 8,100,000	97.6 98.8
3. Male foxhound; weight $20\frac{1}{4}$ kilos.	0	No exercise on this day. Only two deter- minations made.	1,174	100.0	894	100.0						
	6 hrs.		1,127	96.0	911	101.0						
4. Male pointer; weight $20\frac{3}{4}$ kilos.	0	No exercise on this day.	1,402	100.0	683	100.0	88.0	100.0	1,835	100.0		
	1 hr. 5 hrs.		1,536 1,510	109.0 108.0	696 665	102.0 97.0	88.0 87.0	100.0 99.0	1,960 1,890	107.0 103.0		
7. Male mongrel; weight $16\frac{1}{4}$ kilos.	0	No exercise on this day.	998	100.0	723	100.0	114.0	100.0	1,965	100.0		
	1 hr. 5 hrs.		1,020 985	102.0 100.0	620 613	86.0 85.0	105.0 106.0	92.2 93.0	1,722 1,705	88.0 87.0		



TEXT-FIG. 2.

TEXT-FIG. 2. Control findings. The animals were confined, throughout, to small cages.



TEXT-FIG. 3.

— Exercise series.
 - - - Control series.

of dye solution injected. Cell volume shows a marked decrease in four animals, a slight increase in one, and a considerable increase in another. The average for the series is a decrease of 3.2 per cent. Hemoglobin and pigment volume show irregular changes mostly within rather narrow limits. The red count also decreases slightly. The third set of determinations shows irregular variations in all the blood components but within narrow limits, and the average is very close to the normal, taking the results of the first determination as such.

In Table IV, the changes in both series of animals have been compared. Only the average variations in per cent of the initial or "normal" average are there given. These percentage changes are also made the basis of the graphic representation in Text-fig. 3 where

TABLE IV.
Summary of the Average Changes in the Two Series.

Determination.	Plasma volume.		Cell volume.		Hemoglobin.		Pigment volume.		Red corpuscles per c. mm.	
	Exercise series.	Control series.	Exercise series.	Control series.	Exercise series.	Control series.	Exercise series.	Control series.	Exercise series.	Control series.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
First.	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Second.	103.6	104.6	112.3	96.8	108.5	98.6	116.1	99.2	111.0	97.1
Third.	111.7	101.6	109.4	99.0	99.6	98.8	108.4	98.9	92.6	104.5

the curves of the average changes in the two series are superimposed. The comparison demonstrates an initial increase of cell volume and of circulating blood pigment as a result of brief exercise. There is a slight initial increase in plasma volume. During more prolonged exercise the cell volume and circulating blood pigment tend to decrease while a dilution of the blood occurs by increase in plasma volume.

DISCUSSION.

The Plasma Changes.

Previous studies by other workers have shown that considerable reliance may be placed in plasma volume figures obtained by the vital red method when properly carried out. Our findings indicate that

the initial concentration of the blood during exercise is not due to a loss of fluid, since there is no decrease in plasma volume. On the contrary, as stated by Cohnheim⁴ and others of his school, there is a distinct increase, especially when exercise is prolonged. As to the mechanism of this increase, Gross and Kestner⁵ and Cohn¹⁹ have offered considerable evidence to show that the fluid is taken from the muscles. They believe in a close connection between the increase in plasma volume and profuse excretion of sweat, but our results were obtained in dogs, animals which do not sweat in the ordinary sense. However, water is lost by them in considerable quantities in the process of panting.

The Alterations in the Distribution of Red Cells.

The calculation of total cell volume was made on the basis of the percentage volume of cells in the jugular vein blood, the total plasma volume being known. Hemoglobin, pigment volume, and red count are also influenced by changes in the ratio of cells and plasma. The initial increase in total cell volume shown in these experiments cannot have been due to a mere swelling of the cells, since the hemoglobin increased also. It might conceivably result either from a production of new blood corpuscles or a redistribution of those already in the body. The rapidity of the reaction definitely excludes the first possibility. Schneider⁸ has called attention to the similarity of the cell changes during exercise to those taking place in the early stages of acclimatization to high altitudes, and he believed that a reserve supply of corpuscles from the abdominal vessels is thrown into the peripheral blood stream in both instances. Campbell and Hoagland²⁰ have found that during the process of acclimatization to high altitudes, the peripheral blood contains cells in greater concentration than the mesenteric blood. It is therefore quite possible that, under normal resting conditions, the peripheral vessels are less rich in corpuscles and contain a greater per cent of plasma than the abdominal, and that in response to the demands of exercise, a redistribution takes place.

¹⁹ Cohn, E., *Z. Biol.*, 1920, lxx, 366.

²⁰ Campbell, W. A., and Hoagland, H. W., *Am. J. Med. Sc.*, 1901, cxxii, 654.

If we accept the view of Schneider,³ one can readily explain how it was that Cohnheim and his coworkers⁴ found a decrease in per cent of hemoglobin after the prolonged exertion of mountain climbing. They were at high altitudes before starting on these expeditions, and therefore the readjustments due to acclimatization had already increased as far as possible the per cent of cells in the peripheral blood. The increase in plasma volume accompanying exercise would therefore lead at once to dilution of the blood and the fall in per cent of hemoglobin which they actually observed.

The Changes in the Size of Red Corpuscles.

It is not impossible that changes in the size of red cells occur during exercise. A general swelling of the cells could readily lead to an increase in cell volume. However, the close parallelism between the curves of cell volume and pigment volume in the present work indicates that the results are not due to this cause—for were this the cause there would be disproportionate changes in pigment content and cell bulk. The red counts here recorded are too few in number to furnish reliable data as to average cell size. The literature on changes in cell size during exercise is extremely meager. Price-Jones,²¹ working with dried blood smears, has concluded that the cells become larger. The present findings would, in general, tend to confirm this view, for the decrease in cell volume during prolonged exercise is not nearly so great as the decrease in pigment volume. One might account for this by a disappearance of some cells from the circulation and an increase in size of those that remain.

Evidences of Blood Destruction.

It will be noted that after prolonged exercise both total cell volume and pigment volume fall well below the maximum noted after 10 minutes of exercise. The decrease observed in percentage of hemoglobin and in the number of cells per cubic millimeter is less significant, being partly due to dilution. In view of the uncertain factor of cell distribution, it would be unsafe to conclude that blood de-

²¹ Price-Jones, C., *J. Path. and Bact.*, 1919-20, xxiii, 371.

struction occurs during exercise on the basis of the present data alone, although there is no doubt that they are in harmony with such a view. More facts in the matter will be given in later papers.

SUMMARY.

The following changes have been demonstrated to take place in the blood of dogs during exercise.

1. An increase in the per cent of cells and hemoglobin in the blood of the jugular vein occurs early in the course of exercise. It probably results from a redistribution of red corpuscles, with an increase in their proportion in the peripheral blood.

2. As exercise is continued, there is a definite increase in plasma volume.

3. A coincident decrease both in the total cell volume and the pigment volume during prolonged exercise suggests that blood destruction then occurs.

EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

IX. THE RECURRENCE OF 1922.

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PLATES 46 TO 49.

(Received for publication, June 27, 1922.)

INTRODUCTION.

The presence in New York City during the early months of 1922 of an acute respiratory infection that, clinically, resembled more or less the epidemic influenza of the autumn and winter of 1918-19 and the spring of 1920 afforded us an opportunity to repeat and extend the studies of the nasopharyngeal secretions from influenza patients which had resulted in the isolation of *Bacterium pneumosinles*. The reports of the earlier experiments have formed Papers I to VIII of the present series.¹⁻⁸

As an introduction to our observations with material obtained during the 1922 recurrence of influenza, the facts already established may be passed briefly in review, since they form the basis for a comparison of the data recently obtained with the findings in similar experiments during the past 3½ years.

¹ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

² Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 361.

³ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 373.

⁴ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

⁵ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiv, 1.

⁶ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 1.

⁷ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 553.

⁸ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 813.

Summary of Earlier Observations.

Having in mind the possible presence in the nasopharyngeal secretions of influenza patients of an agent whose effects might be noted in animals, we injected the whole or the filtered nasopharyngeal washings intratracheally into rabbits. In eight instances the nasopharyngeal washings were obtained within 36 hours of the onset of the uncomplicated disease. The material from five of these patients during the epidemic wave of 1918-19 and from two during the recurrence of 1920 produced typical clinical and pathological effects in the experimental animals. On the other hand, the active agent was not obtained from twelve influenza patients, the onset of whose illness had occurred more than 36 hours previously, nor from fourteen persons free from influenza during the epidemic and interepidemic periods.¹

The effects produced by the active agent in the experimentally infected animals bore a certain similarity to those observed in epidemic influenza in man. Clinically, the affection was typified by a rise in temperature within 24 to 48 hours, by conjunctivitis, and by a characteristic change in the blood picture—a rapidly developing leucopenia, due mainly to a critical drop in the mononuclear cells. Unless the train of events was interrupted by secondary infection, the rabbits returned to normal in the course of 2 or 3 days. Most of them were killed after 24 to 48 hours, however, for observation of the lung lesions and to obtain material for further passage, and for cultivation. In such instances a typical pathological condition was revealed, consisting of a diffuse hemorrhagic edema of the lungs, with emphysema, and with a peculiar interalveolar cellular exudate, and a similar hemorrhagic involvement of bronchi and trachea, with exfoliation of the necrotic epithelium.¹

When lung tissue containing such lesions was ground and extracted with salt solution, the turbid suspension produced similar effects in other rabbits, and in this way the substance active in the process was transmitted for as many as fifteen passages, indicating the multiplication of a living agent rather than the mere passive transfer of some original material.

This living agent was found to have the following properties.

As it existed in the nasopharyngeal secretions in man, and in the lungs of affected rabbits, it passed through Berkefeld V and N candles, and the filtered material produced the same effects on the blood and in the lungs of rabbits and guinea pigs as the unfiltered material.²

When contained in bits of lung tissue, it withstood the action of a 50 per cent glycerol solution for periods up to 9 months.²

It produced effects upon the pulmonary tissues of rabbits which favored the invasion and infection of the lung with other bacteria, such as the pneumococcus, streptococcus, and *B. Pfeifferi*.³

It led to the development, in experimentally infected animals, of a specific immunity against reinfection with similar material.⁶

Meanwhile, cultivation experiments had shown the presence in the active material of a hitherto undescribed microorganism. This microorganism, which was subsequently named *Bacterium pneumosintes* because of its injurious effect upon the lung, was first observed in November, 1918, in strictly anaerobic cultures of the filtered nasopharyngeal secretions of an influenza patient in the early hours of the disease. Subsequently the same bacterium was isolated from material originally derived from all the seven cases of influenza from which active material was transmitted to rabbits, and from three other cases not so transmitted. The immediate sources of the cultures were the filtered nasopharyngeal secretions of patients or, more frequently, the unfiltered or filtered lung tissue suspensions of affected rabbits. These rabbits had been intratracheally injected with whole or filtered nasopharyngeal secretions, whole or filtered lung suspensions of other affected animals, or the glycerolated lung tissue of such animals. The cultivation experiments were carefully controlled with similar materials from non-influenzal sources. All of the control materials gave negative results.⁴

The primary cultures of *Bacterium pneumosintes* were obtained under strictly anaerobic conditions in a medium composed of sterile human ascitic fluid and a fragment of fresh rabbit kidney. In this medium the microorganism developed slowly, producing a faint haze in the region of the kidney fragment that gradually extended to a depth of about 3 cm. by the 8th day and in the course of 2 weeks settled to the bottom of the tube, leaving a clear, faintly opalescent, supernatant fluid.

In stained films, *Bacterium pneumosintes* appeared as a minute bacilloid body of regular morphology, measuring 0.15 to 0.3 micron in the long axis. Usually solitary, the bacteria were often found in diplo form, and occasionally in short chains of three or four members. All of the strains decolorized uniformly by Gram's method. They were stained with some difficulty by the usual basic dyes.

The intratracheal injection, in rabbits and guinea pigs, of mass cultures of *Bacterium pneumosintes* in the earlier generations induced effects on the blood and lungs of these animals which could not be distinguished from those obtained with the active agent of the nasopharyngeal secretions of influenza patients. In addition to this identity of source and of pathogenic effect in animals, *Bacterium pneumosintes* exhibited the other qualities characteristic of the active agent. Thus the microorganism passed Berkefeld V and N filters even in remote generations. Protected by the lung tissue of rabbits affected by the injection of mass cultures, it withstood glycerolation for a period of 9 months. Infection with *Bacterium pneumosintes* reduced the resistance of the pulmonary tissues of rabbits to infection with other bacteria, such as *Pneumococcus* Type IV and *B. Pfeifferi*.⁴ Finally, by cross-immunity experiments, the antigenic identity of the various strains of *Bacterium pneumosintes* with each other and with the active influenzal agent was completely established.

Before methods had been found for the cultivation of *Bacterium pneumosintes* in simplified media, the opportunity was lost to examine for specific antibodies the blood serum of persons convalescent from influenza or of most of the animals

which had recovered from the experimental infection. Later, methods of cultivation were devised which did not interfere with the detection of specific antibodies.^{8,9} Rabbits intravenously injected with such cultures produced antibodies which were recognized by precipitation, agglutination, complement fixation, and phagocytic tests, and animals injected with glycerolated lung tissue containing the active agent yielded serum that specifically agglutinated *Bacterium pneumosintes*. It is significant that the four available strains of *Bacterium pneumosintes*, three from 1918-19 and one from 1920, showed identical antigenic characters by serological and immunological tests.

More recent experiments on methods of cultivation⁸ have shown that *Bacterium pneumosintes* can be grown on media consisting of meat infusion peptone broth, or nutrient agar, as a base, enriched with fresh animal or vegetable tissue, fresh defibrinated rabbit blood, or by the growth of other organisms. Surface colonies have been obtained on blood agar plates in the Brown anaerobic jar.¹⁰ The plates have proved especially useful for the purification of contaminated cultures and for the demonstration of viable organisms in sparse primary growths in the ascitic fluid-rabbit kidney medium. When grown on media containing nutrient broth, and especially in the presence of dextrose, *Bacterium pneumosintes* has developed larger bacillary forms up to 1 micron in length. The identity of these microorganisms with the original strains has been proved by serological reactions and by their reversion to the minute forms on transfer to the original medium.

Animal Transmission and Cultural Studies with Recent Materials.

As a result of the experiments outlined above, we had facilities for animal transmission and cultural studies at hand during the recent outbreak of clinical influenza in January and February of the present year and were able to proceed without the loss of time that was formerly spent in an empiric search for methods.

This outbreak of influenza enabled us to examine the nasopharyngeal secretions of nine persons who had within a few hours developed symptoms characteristic of the disease. The following protocol is cited as a typical example of the cases studied.

Case 36.—Adult male. Feb. 9, 1922. Illness began at night with chilly sensations, a thin, clear secretion from the nose, fever, and pains in the joints and muscles, especially those of the back and legs. Feb. 10. Remained in bed. Temperature 38.3°C., marked coryza, intense injection of conjunctivæ, dry cough. Physical examination showed no pulmonary involvement. Leucocytes

⁹ Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 635.

¹⁰ Brown, J. H., *J. Exp. Med.*, 1921, xxxiii, 677; 1922, xxxv, 467.

4,000, of which 3,920 were polymorphonuclear neutrophils, and 80 were mononuclears. On this day, 12 hours after onset, nasopharyngeal washings were obtained for examination. Feb. 11. Patient's condition improved. Feb. 12. Temperature normal. Recovery uneventful.

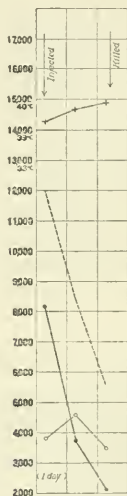
Eight other patients whose nasopharyngeal secretions were obtained from 6 to 36 hours after onset exhibited symptoms similar to those described. The washings from one of these patients stood at room temperature for 24 hours before transmission and cultivation experiments were undertaken. Both failed. Of the remaining eight specimens, successful transmission experiments were initiated with six. The seventh failed to induce the experimental disease in rabbits. The eighth specimen was not injected into animals, but after filtration through a tested Berkefeld candle it yielded a primary growth of *Bacterium pneumosintes* in an anaerobic ascitic fluid-rabbit kidney medium.

The following protocol of a typical animal transmission experiment is reported for comparison with the recorded results of the earlier experiments.¹

Case 28.—The unfiltered nasopharyngeal washings obtained 6 hours after onset were injected intratracheally into Rabbit A.

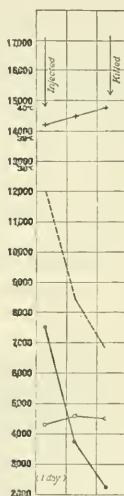
First Passage. Rabbit A (Text-Fig. 1).—Jan. 25, 1922. Leucocytes 12,000, of which 3,840 were polymorphonuclear cells and 8,160 were mononuclear cells. Temperature 39.2°C. Injected intratracheally with 2.5 cc. of unfiltered nasopharyngeal washings of Case 28. Jan. 26. Leucocytes 8,400, of which 3,780 were mononuclears. Temperature 39.7°C. Jan. 27. Leucocytes 5,600, of which 2,128 were mononuclears. Mild conjunctivitis. Temperature 39.9°C. Killed. All organs appeared normal except the lungs. These showed a diffuse edema and emphysema with a number of small hemorrhages scattered over the surface and on the cut sections. There was an absence of pneumonic consolidation. On microscopic examination (Fig. 1) the edema, emphysema, and the small hemorrhages were again revealed. There was a moderate degree of cellular infiltration of the interalveolar strands and about the bronchi and bronchioles, which consisted of mononuclears, polymorphonuclears containing acidophilic granules, and a few cells of the respiratory type. In addition, the interalveolar strands were distended with serum and erythrocytes. The bronchial system was also involved in a hemorrhagic and edematous process. The epithelium was necrotic in some areas and the lumen was filled with serum, leucocytes, erythrocytes, and exfoliated necrotic epithelial cells. Impression smears of the lung stained by Gram's method revealed numerous mononuclears and alveolar cells, but no bacteria. Aerobic cultures were free from growth.

In Giemsa-stained preparations of sections of the lung groups of minute organisms indistinguishable morphologically from *Bacterium pneumosintes* were noted in the bronchial exudate. Some were free and others in the different stages of phagocytosis by polymorphonuclears and monocytes (Fig. 2). In the ciliary margin of the bronchial epithelium similar clumps of these microorganisms were seen (Fig. 3).



TEXT-FIG. 1.

- + — + Temperature.
- - - Total leucocytic count.
- — • Mononuclear cells.
- — ○ Polymorphonuclear cells.



TEXT-FIG. 2.

TEXT-FIG. 1. Effect on the blood count and temperature. The rise in temperature and the depression in the total white blood cell count caused by a deficiency of mononuclears are shown. First rabbit passage of the nasopharyngeal washings from a patient (Case 28) in the early stage of uncomplicated epidemic influenza (1922). Intratracheal injection.

TEXT-FIG. 2. Effect on the blood count and temperature. The rise in temperature and the depression in the total white blood cell count caused by a deficiency of mononuclears are shown. Rabbit passage of *Bacterium pneumosintes* from a culture, in the third generation, originally derived from a rabbit inoculated with the washings from another influenza patient (Case 36).

In the present series of experiments animal passage failed after the first transfer in one instance. The other transmission experiments were discontinued after the second to fourth transfers.

The effects produced by the intratracheal injection into rabbits of these specimens of unfiltered nasopharyngeal secretions from influenza patients appeared to us to be identical with those observed during the former epidemic waves. The active agent showed other familiar qualities also. It survived the action of 50 per cent glycerol for 4 months (the longest period yet tested) and passed Berkefeld V and N filters, producing characteristic lesions in rabbits injected with the filtrate.

As controls for these experiments, we have negative transmission experiments with the nasopharyngeal secretions of three persons not suffering from influenza. One of these had a mild head cold which ran a typical course; the others were normal.

Because of the identity of the active agent with *Bacterium pneumosintes* in former experiments, our endeavor was directed mainly toward the demonstration of a filterable anaerobic microorganism in the material obtained during this recurrence of influenza. Filtered nasopharyngeal secretions from each of the nine influenza patients and from ten other persons were inoculated into ascitic fluid and rabbit kidney tubes under a vaseline seal, and spread upon blood agar plates in an anaerobic jar. The lung tissues of affected rabbits were likewise cultured in the fluid medium, and sometimes smeared on the anaerobic plates. When no organisms or only a few minute and questionable bodies could be demonstrated with the microscope in fluid cultures, material from these cultures was spread on blood plates for the possible observation of colony formation.

By these methods there were obtained cultures of strictly anaerobic, filter-passing, Gram-negative organisms in material derived from four of the six influenza patients from whom an active agent had been transmitted to rabbits, from a seventh influenza patient whose nasopharyngeal washings were not injected into animals, and from four of ten other persons not suffering from influenza. A survey of these microorganisms showed that *Bacterium pneumosintes* is not the only anaerobic, filter-passing, Gram-negative organism to be found in the human respiratory tract. From one of the influenza patients and from

the four non-influenzal controls which yielded positive cultures, other bacteria, not pathogenic for rabbits and not *Bacterium pneumosintes*, were obtained. A preliminary description of these other bacteria is appended to this paper.

On the other hand, we have identified as *Bacterium pneumosintes* the cultures derived from three of the six influenza patients, enumerated above, whose nasopharyngeal secretions were pathogenic for rabbits, and from the seventh patient, whose secretions were not used in a transmission experiment. No cultures of *Bacterium pneumosintes* were obtained from the controls.

It is noteworthy that only one strain of *Bacterium pneumosintes* was obtained directly from the filtered nasal washings. The others were derived from lung tissue after rabbit passage. In this connection the relative value of these two immediate sources may be indicated. Nasopharyngeal secretions require filtration before direct cultivation in fluid media and much of the active material, mixed with mucus and epithelial debris, is retained on the filter and lost. It is probable that conditions are only occasionally favorable for the primary development of *Bacterium pneumosintes* in artificial culture. As with the pneumococcus a relatively large inoculum is usually required to initiate growth. Moreover, if the first inoculations fail the entire opportunity is lost. But when the whole nasopharyngeal washings are injected into rabbits, the larger primary inoculum is further augmented by active multiplication in the pulmonary tissues, and in those favorable instances in which contaminating bacteria are suppressed, a material rich in *Bacterium pneumosintes* in pure culture is available for artificial cultivation either directly or after filtration. Moreover, through continued rabbit passage repeated opportunities are afforded for cultivation experiments and sooner or later they usually succeed. We feel that although direct cultivation of filtered nasopharyngeal secretions may be a suitable method for the demonstration of other anaerobic filter-passing microorganisms, a negative experience in the cultivation of *Bacterium pneumosintes* without the aid of animal passage is of little significance.

Identification of New Strains of Bacterium pneumosintes.

The identification of the new strains of *Bacterium pneumosintes* was made on morphology, cultural characters, filterability, typical pathogenicity for rabbits, resistance to glycerol, reduction of resistance to secondary infection, and serological and immunological reactions.

Not all of these tests have been made with each strain, but each has been definitely identified by means of a combination of them, and all

have been agglutinated specifically by sera made with the 1918-19 strains as antigen. These experiments also served as a review and check on the characters of *Bacterium pneumosintes* as formerly observed, and confirmed our reported findings. For these reasons they may be described more or less in detail.

Morphological and Cultural Characters.—The morphology and cultural characters of the new strains are in all respects identical with those of the strains obtained in 1918-19 and 1920. Photographs of Strain 16,⁴ from the 1918-19 epidemic, and Strain 36, obtained in February, 1922, may be compared in Figs. 5 and 6. Colonies of these strains on anaerobic blood agar plates are shown in Figs. 8 and 9.

Filterability.—Mention has already been made of Strain 32, which was obtained by direct cultivation of the filtered nasopharyngeal washings from Case 32. Other new strains, obtained from rabbit lung tissues, have passed Berkefeld V candles and initiated growth in culture media.

Pathogenicity.—So far all of the new strains tested (first to eighth generation) have retained their pathogenicity for rabbits. With one strain the process of intratracheal injection, followed by the characteristic infection and recovery of the organism from the lung tissue, has been repeated five times. The three other strains have been recovered in this manner twice. A typical protocol illustrates the clinical and pathological results of intratracheal injection of the strain of *Bacterium pneumosintes* derived from Case 36.

Rabbit A.—Mar. 15, 1922. Leucocytes 12,000, of which 7,680 were mononuclear cells. Temperature 39.2°C. Injected intratracheally with 3 cc. of a suspension of washed mass culture⁴ of *Bacterium pneumosintes*, the third generation of Strain 36. Mar. 16. Leucocytes 8,400, of which 3,780 were mononuclear cells (Text-fig. 2). Temperature 39.5°C. Mild conjunctivitis. Mar. 17. Leucocytes 6,800, of which 2,244 were mononuclear cells. Temperature 39.8°C. Mild conjunctivitis. Killed. Observable lesions were confined to the lungs and trachea. The lungs were the site of a diffuse hemorrhagic edema and emphysema without any obvious consolidation. Microscopically, the hemorrhages, edema, and emphysema were noted as well as the typical cellular exudation and bronchial lesions similar to those found in the lungs of the rabbit injected with the nasopharyngeal secretions from an influenza patient, and described above (Fig. 7).

In Giemsa-stained preparations of the lung tissue small groups of microorganisms, indistinguishable from *Bacterium pneumosintes*, were found in the ciliary layer

of the bronchial epithelium. Aerobic cultures of the lung tissue showed no growth. Anaerobic cultures in ascitic fluid-rabbit kidney medium yielded pure growths of *Bacterium pneumosintes*.

Locus of Bacterium pneumosintes in Affected Lungs.—We have not heretofore reported direct observations on the presence of *Bacterium pneumosintes* in the lungs of infected rabbits. Frequently in the course of the earlier studies, in impression smears or sections of affected rabbit lungs, stained by Gram's method, with Löffler's methylene blue, or with methylene blue and eosin, minute but questionable bodies had been made out. In view of their inconstancy and the difficulty of demonstrating them with the stains employed, we had not been willing to accord them diagnostic significance. Recently in microscopic sections stained by Giemsa's method, minute blue- or violet-stained bodies, morphologically identical with *Bacterium pneumosintes*, have been found repeatedly in scattered groups in the affected pulmonary tissues. Their usual site is deep in the ciliary margin of bronchial epithelium (Figs. 3 and 4). Less often the bodies are found in mucous and serous exudate between the cilia and in the lumen of the bronchioles, where they are in process of phagocytosis by polymorphonuclear cells and monocytes (Fig. 2). Infrequently they have been found in the interalveolar strands, where they are usually surrounded by an inflammatory mononuclear cell reaction.

These minute and characteristic bodies, which have not been observed in normal rabbit lung tissue, have been clearly made out in various sections of the pulmonary tissues of six rabbits injected with the active agent of the nasopharyngeal secretions of influenza patients. From three of these rabbits pure cultures of *Bacterium pneumosintes* were obtained. The incidence and distribution of these bodies in the lesions caused by *Bacterium pneumosintes*, and their morphological and tinctorial similarity to this microorganism lead us to suppose that they are *Bacterium pneumosintes*.

Reduction of Resistance to Secondary Infection.—A peculiar and significant property of the strains of *Bacterium pneumosintes* obtained in 1918-19 and 1920 was their effect in reducing the resistance of the lung tissues of experimentally infected rabbits to invasion by other bacteria.⁵ Some of the recently isolated strains have been tested in a similar manner and with similar results. For example, after

repeated trials had shown that 3 million pneumococci of an atypical Type II strain, injected intravenously, rapidly disappeared from the blood stream and produced no lesions in rabbits, the tested dose was used in controlled experiments such as the following.

Rabbit A.—May 16, 1922. Leucocytes 6,300, of which 3,090 were mononuclear cells. Temperature 39.2°C. Injected intratracheally with 3 cc. of a saline solution suspension of *Bacterium pneumosintes*, Strain 34, third generation in mass culture.⁴ May 17. Leucocytes 6,000, of which 1,200 were mononuclear cells. Mild conjunctivitis. Temperature 39.5°C. Injected intravenously with 1 cc. of a saline solution suspension containing 3 million pneumococci, atypical Type II. May 18. Leucocytes 5,600, of which 2,520 were mononuclear cells. Conjunctivitis. Temperature 41.2°C. May 19. Leucocytes 18,800, of which 11,280 were polymorphonuclear cells and 7,520 were mononuclears. Conjunctivitis. Temperature 40.7°C. Blood taken for culture. Killed. The lower right lobe was voluminous, dark, and resistant to the knife. Section revealed a dark and congested cut surface. Fibrinopurulent material exuded from the bronchi. Microscopically, the exudate was found to consist mainly of polymorphonuclears with acidophilic granules and a few mononuclear and respiratory type cells with fibrin and serum. Impression smears of the lung stained by Gram's method showed numerous polymorphonuclear cells containing phagocytosed pneumococci. Free pneumococci seen also. The blood culture and the aerobic and anaerobic cultures of the lungs yielded pure growths of the pneumococcus. Diagnosis: pneumococcus septicemia and pneumonia.

Precisely similar results were obtained with the streptococcus and *Bacillus pfeifferi*. These experiments will be described in detail in the next paper of this series.

Serological Reactions.—The first anti-*pneumosintes* rabbit serum produced was obtained by five weekly injections of a suspension of live organisms (Strain 11) and showed an agglutination titer of 1:160 to 1:320 against the homologous strain.⁷ Other sera, produced with live or killed cultures of Strains 16, 17, and 26, have shown a dilution limit of 1:16 to 1:32 for complete agglutination, even after prolonged immunization. We are inclined to believe that, aside from individual variation in rabbits, the relatively low titer of immune serum to be obtained with *Bacterium pneumosintes* is characteristic of the organism.

For diagnostic purposes this low titer of the anti-*pneumosintes* serum is not objectionable, since the reactions at dilutions of 1:8 and 1:16 are sharp and specific. We have never seen *Bacterium pneu-*

mosintes agglutinate in normal rabbit serum, even undiluted, and the bacterium shows no tendency to spontaneous flocculation in salt solution, remaining in smooth suspension for 24 to 48 hours. Dr. De Kruif, who has done some unreported experiments on the acid agglutination zone of *Bacterium pneumosintes*, informs us that it flocculates out in an unusually narrow zone, lying between pH 3.0 and 3.6, and he infers from this that *Bacterium pneumosintes* is a stable microorganism, not easily agglutinable by immune serum or other chemical or physical agents.

TABLE I.

Agglutination Tests with Old (Strains 17 and 26) and New Strains of Bacterium pneumosintes.

Strain No.	Immune Serum D.				Normal rabbit serum.				Salt solution.
	Dilutions.				Dilutions.				
	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	
17	+++	+++	+++	+++	—	—	—	—	—
26	+++	+++	+++	+++	—	—	—	—	—
32	+++	+++	+++	+++	—	—	—	—	—
34	+++	+++	+++	++	—	—	—	—	—
36	+++	+++	+++	+++	—	—	—	—	—
40	+++	+++	+++	+++	—	—	—	—	—

In Table I are given the results of an agglutination test with old and new strains of *Bacterium pneumosintes* against immune rabbit serum produced with one of the old strains. The serum was obtained May 6, 1920, from a rabbit intravenously injected with living cultures of Strain 11. The bacterial suspensions for agglutination were grown in *Bacillus coli* broth,⁸ centrifuged, taken up in salt solution, killed by heat at 56–60°C. for 30 minutes, and standardized by opacity. The tests were set up in successive dilutions in capsules,¹¹ incubated at 55°C. for 1 hour, and stored in the ice box over night before macroscopic examination.

¹¹ Gates, F. L., *J. Am. Med. Assn.*, 1921, lxxvii, 2054.

As a further test of specificity, two of the old strains derived from Cases 16 and 17¹ and three of the new strains from Cases 32, 34, and 36 were examined for their capacity to absorb specific agglutinins.

Suspensions of the absorbing strains, of ground glass opacity, were distributed into two sets of small test-tubes in lots of 1 cc. These tubes were then centrifuged and the supernatant fluid was carefully pipetted off from the compact sediment. To each of one set of tubes was added 1 cc. of immune serum (made with Strain 16 and obtained Feb. 28, 1922) in a dilution of 1:4, the bacteria were suspended by agitation, and the tubes were incubated for 1 hour at 55°C. After centrifugation, the supernatant serum from the incubated tubes (now absorbed once) was poured on to the sediment of the corresponding culture in the second set of tubes, which were shaken and incubated for the second absorption for 1 hour at 55°C. In this way dilution of the serum was avoided. The serum samples, now twice absorbed, together with an unabsorbed control which had been incubated for 2 hours at 55°C., were then used in the agglutination test shown in Table II.

In the table the columns grouped under A show the agglutination of the various strains in the unabsorbed control serum. In Group B each absorbed serum sample was tested against its own absorbing strain. The tests show that absorption of specific agglutinins had occurred but was not complete. In Group C the serum samples, absorbed with the various strains, were tested against the homologous strain, No. 16. All of the strains had removed specific agglutinins for the homologous strain, but again, absorption was evidently not complete with the amounts of bacteria employed. Finally, in Group D the serum was (incompletely) absorbed with the homologous strain, which removed part of the agglutinins for all of the other strains.

Numerous other agglutination tests with the new strains against serum made with the old ones, and *vice versa*, have proved the antigenic identity of the new strains with the old, and among themselves. If antigenic differences or variations exist by which the strains isolated in 1918-19 and 1920 can be differentiated from those obtained in 1922, we have so far failed to discover them.

Immunological Reactions.—Because the old strains, during long cultivation, had lost their pathogenicity for rabbits, we were able to carry out cross-immunity tests in one direction only; namely, to discover whether injections of the old strains protect against subse-

TABLE II.

Absorption Tests with Old (Strains 16 and 17) and New Strains of Bacterium pneumosintes.

Group A.					Group B.				
Absorbing strains.	Strains agglutinated.	Unabsorbed control serum.			Absorbing strains.	Strains agglutinated.	Absorbed serum.		
		Dilutions.					Dilutions.		
		1:8	1:16	1:32			1:8	1:16	1:32
Not absorbed.	16	+++	+++	+++	16	16	++	—	—
	17	+++	+++	+++	17	17	+++	+	—
	32	+++	+++	+++	32	32	+	—	—
	34	+++	+++	+++	34	34	+	+	—
	36	+++	+++	+++	36	36	++	+	—

Group C.					Group D.				
Absorbing strains.	Strains agglutinated.	Absorbed serum.			Absorbing strains.	Strains agglutinated.	Absorbed serum.		
		Dilutions.					Dilutions.		
		1:8	1:16	1:32			1:8	1:16	1:32
17	16	+++	—	—	16	17	+++	++	+
32	16	+++	+	—	16	32	++	+	—
34	16	+++	+	—	16	34	+	—	—
36	16	+++	+	—	16	36	+	—	—

quent infection with the new ones. Two such experiments have been performed and have indicated this protection, as the following protocol shows.

Rabbit A.—Apr. 11, 1922. Injected intratracheally with 3.5 cc. of suspension of centrifuged sediment consisting of live *Bacterium pneumosintes*, Strains 16 and 26, derived from the 1918-19 and the 1920 waves, and grown in *B. coli* broth.⁸ These cultures were at this time not pathogenic for rabbits.⁴ Apr. 24. Leucocytes 12,600, of which 5,544 were mononuclear cells. Temperature 39.7°C. Bled from ear vein for agglutination test. Injected intratracheally with 3 cc. of washed sediment of live mass culture⁴ of *Bacterium pneumosintes*, Strain 34, derived by rabbit passage from Case 34 of the 1922 wave, in the fourth generation. Apr. 25. Leucocytes 12,000, of which 5,760 were mononuclears. Temperature 39.5°C. Conjunctivæ normal. Apr. 26. Leucocytes 10,800, of which 5,724 were mononuclear cells. Temperature 39.5°C. Conjunctivæ normal. Killed. All organs, including lungs, normal. Aerobic and anaerobic cultures of lungs were free from growth.

Rabbit B (Normal Control).—Apr. 24, 1922. Leucocytes 9,000, of which 4,500 were mononuclears. Temperature 39.2°C. Inoculated with the recent living culture of *Bacterium pneumosintes* precisely as in the case of Rabbit A. Apr. 25. Leucocytes 4,800, of which 1,536 were mononuclear cells. Temperature 39.2°C. Conjunctivitis. Apr. 26. Leucocytes 4,600, of which 1,472 were mononuclears. Temperature 39.5°C. Conjunctivitis. Killed. Lungs showed the hemorrhagic edema, emphysema, peculiar cellular exudation, and bronchial lesions characteristic of the action of *Bacterium pneumosintes*. Aerobic cultures of lungs were free from growth; anaerobic culture yielded a pure growth of *Bacterium pneumosintes*.

We have already demonstrated⁶ the presence of agglutinins against *Bacterium pneumosintes* after intratracheal injection of the active influenzal agent with which the rabbit transmission experiments were carried out. In a similar test the serum from Rabbit A, obtained 13 days after the first intratracheal inoculation with the old strains, agglutinated both the old and recent strains of *Bacterium pneumosintes*. The immunity produced by intratracheal injection, even of non-pathogenic strains of *Bacterium pneumosintes*, is therefore demonstrable by serological tests. These observations on protection and antibody formation after intratracheal inoculation agree with those of Besredka,¹² D'Aunoy,¹³ and others.

SUMMARY AND CONCLUSIONS.

From the nasopharyngeal secretions of patients in the early hours of uncomplicated epidemic influenza during the recurrence in New York City in January and February, 1922, we have again obtained an active agent, pathogenic for rabbits, and have identified this active agent as *Bacterium pneumosintes*. Four new strains of this micro-organism have been isolated in pure culture and identified with the 1918-19 and 1920 strains on morphological, cultural, and serological grounds. All of the significant characteristics of the old strains, including their effect upon the resistance of the lungs of rabbits to secondary invasion with other bacteria, have been noted in the new strains, which thus have served to confirm and extend our original observations.

¹² Besredka, A., *Ann. Inst. Pasteur*, 1919, xxxiii, 882; 1920, xxxiv, 361.

¹³ D'Aunoy, R., *J. Infect. Dis.*, 1922, xxx, 347.

Addendum.

Reference has already been made in this paper to the discovery of other anaerobic, filter-passing, Gram-negative microorganisms, not *Bacterium pneumosintes*, in nasopharyngeal secretions from human sources. Three of the five cultures which proved not to be *Bacterium pneumosintes* were derived from the filtered nasopharyngeal secretions of supposedly normal persons. What the importance of these microorganisms may be, or whether they have any pathogenic significance we are not prepared to suggest. They indicate, however, that the cultural methods recently employed in these studies may lead to the isolation of a group, or groups, of hitherto undescribed inhabitants of the upper respiratory tract. Our observations may therefore be reported, even though fragmentary, as an indication of interesting opportunities in this field of bacteriology.

Although all the five strains of bacteria, not *Bacterium pneumosintes*, and not pathogenic for rabbits, which have been isolated, are strictly anaerobic, decolorize by Gram's method, and have passed tested Berkefeld V or N filters once or repeatedly, they differ culturally and morphologically among themselves and fall into three apparently unrelated groups.

Group I is represented by a slender vibrio which was obtained from the filtered nasopharyngeal secretions of an undoubted case of epidemic influenza from which *Bacterium pneumosintes* was not obtained.

This vibrio (Fig. 10) is approximately 0.15 to 0.2 micron in thickness, and from 0.5 to 2 microns long, with rounded ends and a smooth curve in contour which may approach a half circle. In the early generations in artificial culture media extremely minute forms were observed, but in later generations the larger forms have predominated. The culture was filtered through a tested Berkefeld V candle in the third generation. This microorganism usually takes the bacterial stain faintly and uniformly throughout. Occasionally polar staining has been observed. In fluid media, such as ascitic-kidney, dextrose broth-kidney, or *Bacillus coli* broth under a vaseline seal, it grows first in minute flakes, which soon settle out as an amorphous or flocculent sediment leaving a clear supernatant fluid. On rabbit blood plates, incubated in an anaerobic jar, it forms pin-head, dis-

crete and confluent colonies, without odor, which do not discolor or precipitate the medium (Fig. 11). The colonies are raised, convex, with an entire edge and a grayish translucency. The organism does not emulsify readily and tends to spontaneous flocculation. Serum from a rabbit immune to this organism does not agglutinate *Bacterium pneumosintes*.

Group II is represented by an extremely minute bacillus (Fig. 12) isolated from a patient with acute follicular tonsillitis. Morphologically this microorganism is not unlike *Bacterium pneumosintes*, except that it shows greater variations in length and thickness. So far we have been unable to obtain constant growths in fluid media, although the strain had survived and possibly multiplied during several weeks incubation in anaerobic blood broth. On anaerobic blood agar plates it forms extremely minute colonies, well shown in Fig. 13. In microscopic tests with the surface growth on blood agar this microorganism has failed to show any agglutination in anti-*pneumosintes* serum. It is not spontaneously agglutinable.

A third group of organisms, differing from the other two, and possibly among themselves, is found in three strains of filter passers obtained from normal throats. Morphologically these strains are also bacillary, with wide variations between the longest and the shortest forms (Fig. 14). They, too, have failed to grow consistently in fluid media, and have been maintained for many generations on anaerobic blood agar plates. The characteristic colonies of these organisms are shown in Fig. 15. A sharp cone is surrounded by a plateau with an irregular edge. These colonies are slightly brownish in color and can thus be distinguished by transmitted light. The representatives of this group flocculate in normal rabbit serum.

These fragmentary observations are reported not because of any supposed relation to *Bacterium pneumosintes*, but as an indication that other microorganisms, which must be differentiated from *Bacterium pneumosintes*, may be encountered under similar conditions. The isolation of these other bacteria has depended upon the use of anaerobic blood agar plates. The failure of all but one of them to grow in fluid media and the fact that they are not pathogenic for rabbits, under the conditions of our experiments, show why they were not encountered during the earlier periods of study, before the blood plates were used.

EXPLANATION OF PLATES.

PLATE 46.

FIG. 1. Microscopic appearance of a section of the lung of a rabbit injected intratracheally with the nasopharyngeal secretions from a patient with early influenza (Case 28). The edema, emphysema, and, in the upper portion, hemorrhage are shown. To be noted also are the distention of blood vessels with blood, the bronchial lesion (lumen filled with serum, leucocytes, and exfoliated necrotic epithelium), and the interalveolar cellular exudate. $\times 250$.

PLATE 47.

FIG. 2. Giemsa-stained section of the lung from a rabbit injected intratracheally with the nasopharyngeal secretions from another influenza patient (Case 30). Lumen of a bronchus. Microorganisms indistinguishable from *Bacterium pneumosintes* are seen at points marked A, mixed with precipitate at B, phagocytosed by polymorphonuclear cells at C, and by an endothelial leucocyte, or monocyte, at D. The ciliary margin is indicated by E. $\times 1,000$.

FIG. 3. Giemsa-stained section of the lung shown in Fig. 1. A clump of microorganisms indistinguishable from *Bacterium pneumosintes* is seen deep in the ciliary margin. Aerobic cultures of this tissue were free from growth; anaerobic cultures yielded *Bacterium pneumosintes*. $\times 1,000$.

FIG. 4. Giemsa-stained section of the lung from a rabbit intratracheally injected with a mass culture of *Bacterium pneumosintes*, Strain 34, showing microorganisms in the ciliary layer of a bronchus. $\times 1,000$.

FIG. 5. Stained preparation of *Bacterium pneumosintes*, Strain 16, from the 1918-19 epidemic, in fresh tissue dialysate medium.⁹ Cultivated artificially for $2\frac{1}{2}$ years. To be compared with Fig. 6. $\times 1,000$.

FIG. 6. Stained preparation of *Bacterium pneumosintes*, Strain 36, from the 1922 wave, in fresh tissue dialysate medium. Cultivated artificially for 2 months. To be compared with Fig. 5. $\times 1,000$.

PLATE 48.

FIG. 7. Microscopic appearance of a section of the lung of a rabbit injected intratracheally with a culture of *Bacterium pneumosintes*, Strain 36. To be noted are the lesions similar to those shown in Fig. 1. $\times 250$.

PLATE 49.

FIG. 8. Colonies of *Bacterium pneumosintes*, Strain 16, from the 1918-19 wave, grown anaerobically on the surface of blood agar. $\times 10$.

FIG. 9. To be compared with Fig. 8. Similar colonies of *Bacterium pneumosintes*, Strain 36, from the 1922 wave. $\times 10$.

FIG. 10. Showing the morphology, after repeated subplants, of anaerobic, filter-passing, Gram-negative bacteria of Group I. From a culture in ascitic fluid-fresh tissue medium. $\times 1,000$.

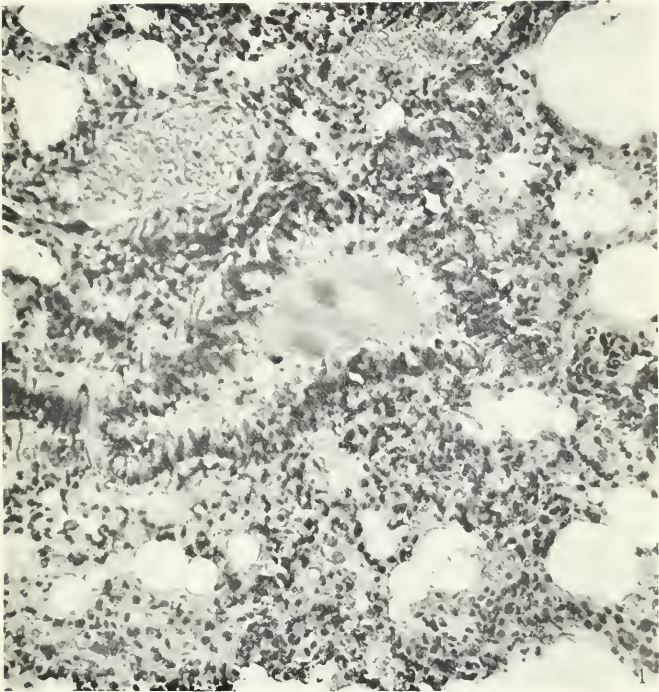
FIG. 11. Colonies of the culture shown in Fig. 10, grown anaerobically on the surface of blood agar. $\times 10$.

FIG. 12. Morphology of anaerobic, filter-passing, Gram-negative bacteria of Group II. $\times 1,000$.

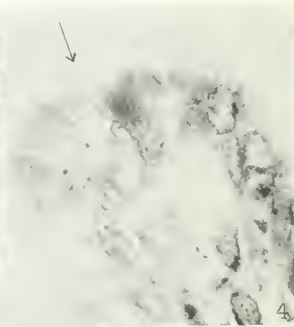
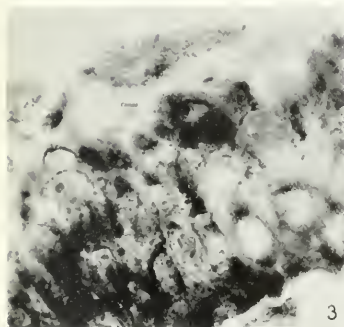
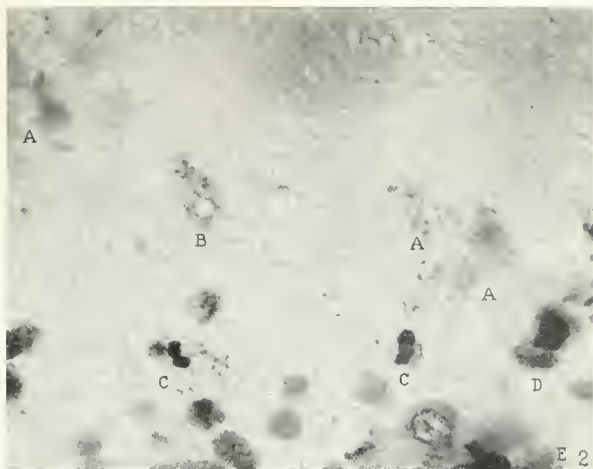
FIG. 13. Colonies of the culture shown in Fig. 12, grown anaerobically on the surface of blood agar. $\times 10$.

FIG. 14. Anaerobic, filter-passing, Gram-negative bacteria of Group III. $\times 1,000$.

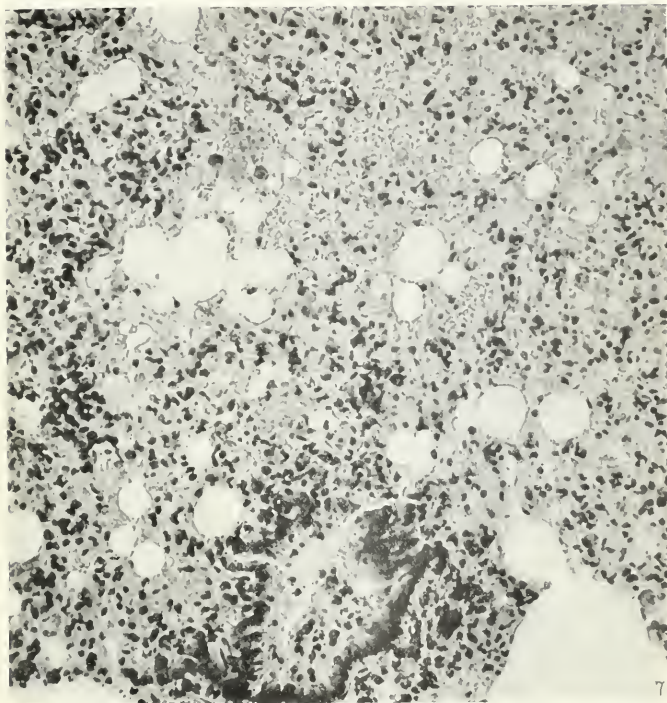
FIG. 15. Colonies of the culture shown in Fig. 14, grown anaerobically on the surface of blood agar. $\times 10$.



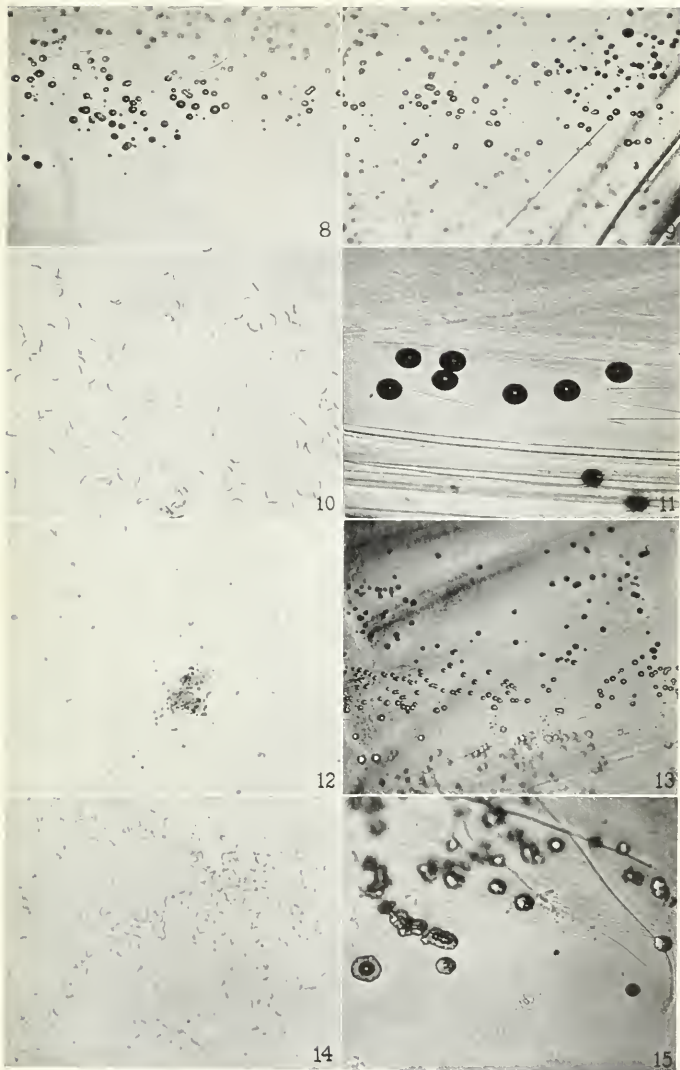
(Olitsky and Gates: Nasopharyngeal secretions from influenza. IX.)



(Olitsky and Gates: Nasopharyngeal secretions from influenza. IX.)



(Olitsky and Gates: Nasopharyngeal secretions from influenza. IX.)



(Olitsky and Gates: Nasopharyngeal secretions from influenza. IX.)

DIFFERENCES BETWEEN MITOCHONDRIA AND BACTERIA.

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The suggestion that the granular and thread-like bodies, which are called mitochondria and are known to occur in almost all active cells from protozoa to man and from fungi to angiosperms, are in reality symbiotic microorganisms, would, if verified, exercise a profound influence in biology and medicine. Yet it has been repeatedly advanced in the literature.

First in 1890, Altmann¹ published a detailed account of his researches made with the help of a new method of fixation in a mixture of equal parts of 5 per cent potassium bichromate and 2 per cent osmic acid, staining in aniline fuchsin and differentiating in picric acid. By this technique the existence of mitochondria (and other granulations) was revealed in many different kinds of cells. To designate them he introduced the term bioblasts, indicating his belief that they are elementary microorganisms responsible for vital phenomena, embedded in a lifeless ground substance; but since he presented no more convincing evidence than a certain resemblance in form between the granules and bacteria, his somewhat involved theoretical considerations were not well received. They came, moreover, at a time when attention was directed toward the nucleus through studies in heredity, and the tremendous impetus of Fischer's² studies on the synthesis of the protein molecule lent encouragement to the view that vital phenomena might soon receive their explanation in terms of physics and chemistry. It is not surprising, therefore, that Altmann's really valuable objective findings were forgotten and investigations along this line received a decided setback.

Again in 1918 Portier³ published detailed investigations purporting to show that mitochondria are symbiotic microorganisms, for which he proposed the term *symbiotes*. His contention was based upon a very careful review of the

¹ Altmann, R., *Die Elementarorganismen und ihre Beziehungen zu den Zellen*, Leipsic, 1890.

² Fischer, A., *Fixierung, Färbung und Bau des Protoplasmas*, Jena, 1899.

³ Portier, P., *Les symbiotes*, Paris, 1918.

literature, but his comparison between mitochondria and bacteria is lacking in directness. For details on the mitochondrial side he has relied upon information given by others. He has not compared the morphology and staining reactions of mitochondria and bacteria occurring side by side within the same piece of tissue. His view has been supported by his colleague Bierry⁴ and very severely criticized on general grounds by Regaud,⁵ Guilliermond,⁶ and Laguesse,⁷ who, however, did not subject the problem to the test of a direct comparison.

More recently Wallin,⁸ in 1922, has published observations upon the staining of bacteria with mitochondrial methods and upon the reactions of bacteria to chemical treatment which have led him independently to advance an hypothesis, like that of Portier, according to which all mitochondria are symbiotic bacteria. His is the first attempt to make a detailed microchemical study, but, like Portier,³ the comparison which he institutes is indirect, for, obviously, the application of mitochondrial methods to bacteria means but little unless they are at the same time applied to mitochondria. From his account as it stands, it would be possible to make out a fairly strong case against the validity of his contention. For example, he notes the fact that alcohol is given as a solvent for mitochondria;⁹ he also records the observation that human tubercle bacilli are not destroyed by immersion for 5 hours in 95 per cent alcohol;¹⁰ yet he emphasizes a similarity in reaction between mitochondria and bacteria.¹¹ The variable resistance of bacteria to the ingredients of mitochondrial fixatives, which he has used separately and in such dilution as to produce extensive ionization, indicates further dissimilarity. One would hardly expect any traces of mitochondria to remain (as he finds the bacteria do) after treatment for 6 hours with 0.5 per cent acetic acid, with 1 per cent formalin, or with 0.5 per cent potassium bichromate.

Although the evidence presented by Altmann,¹ Portier,³ and Wallin⁸ is not convincing, certain inquiries which we have received indicate that the discussion provoked may easily lead investigators from the main problem, which is, we take it, the elaboration of new methods for the determination of the chemical constitution of mitochondria as a prerequisite to the study of their rôle in cellular physiology and pathology. Particularly is this true since the problem lies, as it were, between the sciences of cytology and bacteriology, so that but few investigators can be familiar with both sides of the question. In physiology the cell has come to be considered as a vital unit in which it is not profitable to attempt to distin-

⁴ Bierry, H., *Compt. rend. Soc. biol.*, 1919, lxxxii, 131, 312.

⁵ Regaud, C., *Compt. rend. Soc. biol.*, 1919, lxxxii, 244.

⁶ Guilliermond, A., *Compt. rend. Soc. biol.*, 1919, lxxxii, 309.

⁷ Laguesse, E., *Compt. rend. Soc. biol.*, 1919, lxxxii, 337.

⁸ Wallin, I. E., *Am. J. Anat.*, 1922, xxx, 203.

⁹ Wallin,⁸ p. 204.

¹⁰ Wallin,⁸ p. 213.

¹¹ Wallin,⁸ p. 221.

guish between living and non-living constituents, as Hopkins¹² clearly stated in 1913. To admit, in terms of the evidence presented, that practically all cells contain elements endowed with independent vitality would force a complete readjustment in our ideas of the organization of living matter and in the application to it of the laws of physics and chemistry. In pathology these views, if verified, would have a distinct bearing upon our conception of the significance of *Rickettsia* bodies in typhus fever, trench fever, and other conditions and would leave us in some doubt regarding the criteria to be employed in the identification of microorganisms the nature of which remains obscure.

Thus far the only reaction claimed to be specific for mitochondria is that of coloration with Janus green B (diethylsafranineazodimethylaniline chloride). Wallin⁹ obtained a coloration of bacteria when this stain was employed in a concentration of 1:10,000 in physiological saline solution and uses this observation as a basis for his contention of a similarity between mitochondria and bacteria. But the comparison which he has made is not direct because he apparently relied solely upon accounts given in the literature for knowledge of the behavior of mitochondria under similar conditions. Moreover, we feel that the dye should be employed in greater dilution to bring out its specificity, as the experiments which follow will show.

EXPERIMENTAL.

We have accordingly attempted to make a direct comparison of the Janus green reactions of mitochondria in living lymphocytes and of bacteria under exactly the same conditions. Lymphocytes were chosen because, unlike fixed tissue cells, they may be brought under the microscope with very little mechanical manipulation and studied with ease in the living condition. Their reactions to Janus green have also been standardized. *Bacillus proteus*, *Streptococcus hemolyticus*, pneumococcus, *Bacillus megatherium*, *Bacterium pneumosintes*,¹³ and *Bacillus tuberculosis* (human) were selected as widely representative. They afford examples of microorganisms which are filterable and non-filterable, Gram-positive and Gram-negative, acid-resistant and -non-resistant, aerobic and anaerobic, sporulating and non-sporulating, branching and non-branching, capsulated and non-capsulated, pathogenic (for man and laboratory animals) and non-pathogenic, and which differ in morphology, so that their study may be expected to yield a comprehensive answer.

¹² Hopkins, F. G., *Nature*, 1913-14, xcii, 213.

¹³ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 715.

In each case a very small amount of blood from the ear of a rabbit was placed on a slide; to this was added, with a pipette, a small drop of bacterial emulsion and a solution of Janus green B of known concentration in 0.85 per cent saline solution. The pressure of the

TABLE I.

Comparison of the Avidity with Which Mitochondria in Living Cells and Bacteria Take up Janus Green B.

Concentration of Janus green.	Mito- chondria.	<i>B. proteus.</i>	<i>Strepto- coccus hemoly- ticus.</i>	<i>B. mega- therium.</i>	Pneumo- coccus.	<i>Bacterium pneumo- sintes.</i>	<i>B. tuber- culosis.</i>
1:1,000	+			+			
1:2,000	+			+			
1:3,000	+			+			
1:4,000	+			+			
1:5,000	+			+			
1:6,000	+			+			
1:7,000	+			+			
1:8,000	+			+			
1:9,000	+			+			
1:10,000	+	—	+	—	—	+	+
1:20,000	+		+	—		+	—
1:30,000	+		+	—		+	
1:40,000	+		+	—		—	
1:50,000	+		+	—			
1:60,000	+		+	—			
1:70,000	+		—	—			
1:80,000	+			—			
1:90,000	+			—			
1:100,000	+			—			
1:200,000	+			—			
1:300,000	+			—			
1:400,000	+			—			
1:500,000	+			—			

The method employed was first to test the reaction of *B. megatherium* to the various concentrations of the dye. Having determined that a concentration of 1:10,000 failed to stain this microorganism, we then started at this dilution with the other bacteria, continuing until a negative reaction was reached.

cover-glass brought about a sufficient mixture of fluids. By this method the reactions of living organisms and of mitochondria within living cells may easily be compared side by side and under identical conditions. The results are indicated in Table I.

It was soon found that the mitochondria in living lymphocytes surpass all the organisms examined in their affinity for Janus green. They stain intensely in dilutions of 1:100,000 and may even be colored on the addition of one part of Janus green in half a million of salt solution. The actual dilution of Janus green in contact with the cells is considerably greater than is indicated in the table, for we must take into consideration its further dilution through admixture with the blood and the bacterial emulsion. Viewed in a test-tube a column of, for example, 1:300,000 Janus green about 1 cm. in diameter shows very little color, yet when it is applied to lymphocytes the contained mitochondria are able to concentrate it in their substance so that a mitochondrial rod having an area 1 by $0.2\ \mu$ is plainly colored when observed under the oil immersion lens with strong artificial light.

It is safe to say that none of the bacteria examined are colored on the addition of Janus green in a dilution of 1:100,000. Only two—*Streptococcus hæmolyticus* and *Bacterium pneumosintes*—were stained in a concentration of less than 1:10,000. The readings vary somewhat, depending upon the relative volumes of blood, bacterial emulsion, and of Janus green. But the study of living lymphocytes with their mitochondria intensely stained surrounded by organisms which have not taken up any of the dye leaves little room for doubt that in this respect the reactions of mitochondria and bacteria are entirely different.

For a direct comparison in fixed and stained preparations, the pancreas of the rabbit was selected as a tissue in which mitochondria are well known, through many papers^{14, 15} aimed at a determination of their relation to zymogen formation. The same organisms were suspended in saline solution and injected with a hypodermic syringe into the substance of small pieces of this organ (about 3 mm. in diameter), just after its removal from the body. Immediately thereafter the fragments were fixed and stained in a variety of ways. Five rabbits were used. The pancreas of the first received *Bacillus proteus* and *Streptococcus hæmolyticus*; the second, *Bacterium pneumosintes*; the third, *Bacillus megatherium* and pneumococcus; the fourth,

¹⁴ Hoven, H., *Anat. Anz.*, 1910, xxxvii, 343.

¹⁵ Laguesse, E., *Bibliog. Anat.*, 1911, xii, 273.

Bacillus tuberculosis and *Bacterium pneumosintes*; and the fifth was used as a normal control. By this procedure each organism was brought into immediate contact with mitochondria-laden cells with very little traumatism and without the possibility of either of them being modified through a pathological process of adjustment.

Ordinary fixatives in every-day use in cytology and in bacteriology were employed, as follows: 10 per cent formalin; Zenker's fluid with and without acetic acid; 95 per cent alcohol; Giemsa's fluid (saturated aqueous corrosive sublimate 2 parts and absolute alcohol 1 part); Regaud's fluid (3 per cent potassium bichromate 4 parts and commercial formalin 1 part); A. O. B., or Bensley's acetic-osmic-bichromate mixture (2.5 per cent potassium bichromate 16 cc., 2 per cent osmic acid 4 cc., with 2 drops of acetic acid); acetic-sublimate (saturated aqueous corrosive sublimate 25 cc. and acetic acid 1.25 cc.); and Bouin's fluid (saturated aqueous picric acid 15 cc., commercial formalin 5 cc., and acetic acid 1 cc.).

All the tissues with the exception of those fixed in 95 per cent alcohol and Bouin's fluid were treated as follows: fixed over night; rinsed in water and passed to 50 per cent alcohol at 9 a.m.; 70 per cent alcohol at 10 a.m.; 95 per cent alcohol at 11 a.m.; absolute alcohol at 12 noon; half absolute alcohol and xylol at 1 p.m.; xylol at 1.30 p. m.; 60°C. paraffin at 2.30 p.m.; and embedded at 4 p.m.

After fixation in 95 per cent alcohol tissues were passed to absolute alcohol at noon and carried on as indicated, while after Bouin's fluid they were passed to absolute alcohol at 9 a.m.; half absolute alcohol and xylol at 10 a.m.; xylol at 10.30 a.m.; paraffin at 11 a.m.; and embedded at noon, or transferred to absolute alcohol at noon and embedded as indicated above.

All sections were cut 4 μ in thickness and were stained by Giemsa's method (G), with iron-hematoxylin (FeH), and with fuchsin and methyl green (FMG). The results are given in Tables II to V.

These tables show, from the number of negative signs, that the mitochondria are in general less resistant than the bacteria. For example, they are invariably destroyed by treatment in this manner with 95 per cent alcohol and with Bouin's fluid. They are occasionally and imperfectly preserved by fixation in Zenker's with and without acetic acid, in 10 per cent formalin, in sublimate-acetic, and in Giemsa's

TABLE II.

Mitochondria, B. proteus, and Streptococcus hemolyticus.

Tissue No.	Fixation.	Stain.	Mito- chondria.	<i>B. proteus.</i>	<i>Strepto- coccus hemoly- ticus.</i>
381	10 per cent formalin.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
382	Zenker's fluid less acetic.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
383	Giemsa's fluid.	G	—	+	+
		FeH	+	+	+
		FMG	—	+	+
384	95 per cent alcohol.	G	—	+	+
		FeH	—	+	+
		FMG	—	+	+
385	Regaud's fluid.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
386	A. O. B.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
387	Zenker's fluid with acetic.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
388	Sublimate-acetic.	G	—	+	+
		FeH	Tr.	+	+
		FMG	—	—	—
389	Altmann's fluid.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
390	Bouin's "	G	—	+	+
		FeH	—	+	+
		FMG	—	+	+

TABLE III.

Mitochondria and Bacterium pneumosintes.

Tissue No.	Fixation.	Stain.	Mito- chondria.	<i>Bacterium pneumosintes.</i>
381	10 per cent formalin.	G FeH FMG	— + —	+ + +
382	Zenker's fluid less acetic.	G FeH FMG	— + +	+ + +
383	Giemsa's fluid.	G FeH FMG	— Tr. —	+ + +
384	95 per cent alcohol.	G FeH FMG	— — —	+ + +
385	Regaud's fluid.	G FeH FMG	— + +	+ + +
386	A. O. B.	G FeH FMG	— + +	+ + +
387	Zenker's fluid with acetic.	G FeH FMG	Tr. + +	+ + +
388	Sublimate-acetic.	G FeH FMG	— — —	+ + —
389	Altmann's fluid.	G FeH FMG	— + +	+ + +
390	Bouin's “	G FeH FMG	— — —	+ + +

TABLE IV.

Mitochondria, B. megatherium, and Pneumococcus.

Tissue No.	Fixation.	Stain.	Mito- chondria.	<i>B. mega- therium</i>	Pneumo- coccus.
401	10 per cent formalin.	G FeH FMG	— + —	+ + +	+ + +
399	Zenker's fluid less acetic.	G FeH FMG	— + —	+ + +	+ + +
397	Giemsa's fluid.	G FeH FMG	— Tr. —	+ + +	+ + +
403	95 per cent alcohol.	G FeH FMG	— — —	+ + +	+ + +
400	Regaud's fluid.	G FeH FMG	— + +	+ + +	+ + +
395	A. O. B.	G FeH FMG	— + +	+ + +	+ + +
398	Zenker's fluid with acetic.	G FeH FMG	— + +	+ + +	+ + +
402	Sublimate-acetic.	G FeH FMG	— — —	+ + —	+ + —
396	Altmann's fluid.	G FeH FMG	— + +	+ + +	+ + +
404	Bouin's "	G FeH FMG	— — —	+ + +	+ + Faint.

TABLE V.

Mitochondria, B. tuberculosis, and Bacterium pneumosintes.

Tissue No.	Fixation.	Stain.	Mito- chondria.	<i>B. tuber- culosis.</i>	<i>Bacterium pneumo- sintes.</i>
412	10 per cent formalin.	G	—	+	+
		FeH	+	+	+
		FMG	—	+	+
410	Zenker's fluid less acetic.	G	—	+	+
		FeH	+	+	+
		FMG	—	+	+
408	Giemsa's fluid.	G	—	+	+
		FeH	Tr.	+	+
		FMG	—	+	+
405	95 per cent alcohol.	G	—	+	+
		FeH	—	+	+
		FMG	—	+	+
411	Regaud's fluid.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
406	A. O. B.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
409	Zenker's fluid with acetic.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
413	Sublimate-acetic.	G	—	+	+
		FeH	—	+	+
		FMG	—	—	—
407	Altmann's fluid.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
414	Bouin's "	G	—	+	+
		FeH	—	+	+
		FMG	—	+	+

mixtures. This persistence after fixation in Zenker's fluid with acetic acid and in sublimate-acetic is unusual and may be due to the rapidity of dehydration. As one would expect, Regaud's fluid, A. O. B., and Altmann's fluid proved to be the best preservatives for mitochondria. The bacteria, on the other hand, resist the action of all of the ten fluids. In the preparations the distinction between the solubility of mitochondria and bacteria is often very sharp. In a single field bacteria may be seen in close contact with cells completely deprived of mitochondria (by alcohol and Bouin's fluid) although both bacteria and mitochondria have been subjected to precisely the same influences.

There is also a pronounced difference in staining reaction. Although iron-hematoxylin and fuchsin and methyl green stain both bacteria and mitochondria, Giemsa's stain, which is perhaps the best adapted for the demonstration of bacteria, colors the mitochondria little, if at all. The failure of this so called nuclear dye to color mitochondria specifically points to a difference which we must consider to be fundamental between mitochondria and bacteria. The staining of bacteria with basic dyes is usually attributed to the presence in them of chromatin-like materials containing iron. Molisch,¹⁶ particularly, has made many contributions regarding the widespread occurrence of iron in bacteria. But Nicholson¹⁷ has applied the Macallum¹⁸ hematoxylin and hydrochloric acid test for iron to tissues known to contain mitochondria without obtaining any trace of coloration, which is strong evidence of the absence of masked iron in mitochondria.

In respect to their morphology also we feel that our preparations show a noticeable difference between mitochondria and bacteria. While not denying that some types of bacteria exhibit a high degree of pleomorphism and that, in searching through the body, cells may be found in which the mitochondria are much more uniform in appearance than in the pancreas, we refer to the observations of Lewis and Lewis¹⁹ on mitochondria in tissue cultures. These investigators followed

¹⁶ Molisch, H., *Die Pflanze in ihren Beziehungen zum Eisen*, Jena, 1892.

¹⁷ Nicholson, F. M., personal communication.

¹⁸ Macallum, A. B., *Quart. J. Micr. Sc.*, 1896, xxxviii, 175.

¹⁹ Lewis, M. R., and Lewis, W. H., *Am. J. Anat.*, 1914-15, xvii, 339.

the changes in form of mitochondria in living cells and succeeded in demonstrating a plasticity and modifiability far greater than that possessed by any organism with which we are familiar.

DISCUSSION.

From the foregoing experiments it appears that mitochondria show microchemical and tinctorial properties differing from those of bacteria. It seems pertinent, however, to give a definition of bacteria and to indicate the points on which definite information is required before the theories advanced by Altmann, Portier, and Wallin can be considered acceptable.

According to Park and Williams:²⁰ "The properties of bacteria . . . which are fairly constant under uniform conditions and which have been more or less used in systems of classification, are those of spore and capsule formation, motility (flagella formation), reaction to staining reagents; relation to temperature, to oxygen and to other food material, and, finally, their relation to fermentation and disease." To which we may add the property of forming smaller or larger aggregates (colonies), the individuals of which are, however, physiologically independent (Zinsser²¹), and the development of characteristic modes of growth on suitable artificial media.

It must be admitted in all these respects that a great gulf remains between mitochondria and bacteria. In respect to mitochondria definite spores and capsules have not been noted. The mitochondrial blebs which Wallin suggests may be due to fixation bear in our judgment no resemblance to spores. They often contain easily recognizable substances like neutral fat or starch (in plants) or a clear watery chromophobe fluid, and may clearly be seen in living acinus cells of the pancreas. Motility due to flagellar action has not been observed, and certain differences in staining reaction have been noted above. The temperature, oxygen, and food requirements of mitochondria can only be expressed in terms of the requirements necessary for the vitality of the cell as a whole. It seems unsafe to conclude, as Wallin

²⁰ Park, W. H., and Williams, A. W., *Pathogenic microorganisms*, Philadelphia and New York, 7th edition, 1920, 24.

²¹ Zinsser, H., *A textbook of bacteriology*, New York, 5th edition, 1922, 9.

has done, that because certain forms of bacteria cannot be demonstrated by staining after treatment with moist heat at 49°C. for 30 minutes they necessarily resemble mitochondria which are also destroyed by somewhat similar treatment, because many bacteria are much more resistant. No mitochondria, on the other hand, are known to resist a temperature of over 50°C. Neither is there any parallelism between mitochondria and bacteria in their relation to fermentation and to disease. In fact, their tendency is rather to decrease than to increase in diseases of infectious nature. Furthermore, there is no reason to believe that mitochondria possess the power of independent and characteristic growth apart from cells. Experiments in this laboratory²² on the cultivation of kidney, brain, spleen, heart muscle cells, and blood in a variety of fluid and semi-solid media by aerobic and anaerobic methods have failed to reveal the multiplication of any forms at all suggestive of mitochondria.

The suggestion that mitochondria are independent microorganisms rests in our judgment upon no other evidence than a slight similarity in form of substances of about the same size.

CONCLUSION.

A direct comparison of mitochondria and bacteria in the living condition, as well as in permanent preparations, subjected to the same influences, and viewed side by side, reveals microchemical and tinctorial differences which can only be attributed to a fundamental dissimilarity in their chemical constitution.

²² Reported in part by Olitsky (Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 525).

ACTION OF ANTIGEN ON FIBROBLASTS IN VITRO. II.

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(Received for publication, June 10, 1922.)

It is known that a strain of fibroblasts responds to the presence of an antigen in its culture medium by becoming immunized against the action of the antigen.¹ The purpose of this paper is to report further experiments in which the influence of the antigen concentration on the process of immunization of fibroblasts *in vitro* was studied.

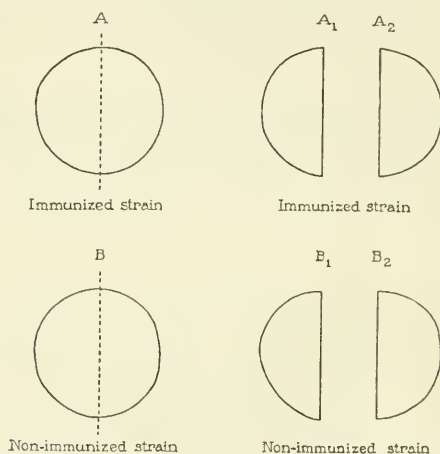
I.

Technique.

The technique was the same as described previously.¹ Dog sera and human ascitic fluid were used as antigen. The antigen was added to chick embryo juice in such a proportion that one volume of the mixture and one volume of chicken plasma would give the desired concentration. Each passage represented 48 hours, after which time the cultures were measured. The experiments were made in the following way. A fragment of tissue taken from a 10 year old strain of fibroblasts was divided into two equal parts. One was cultivated in a medium with antigen (Text-fig. 1, *A*); the other was cultivated in a medium without antigen (Text-fig. 1, *B*) and served as a control. After 48 hours incubation, the experimental and control cultures were divided into two parts. One-half of the experimental culture was transferred to a fresh medium with antigen (Text-fig. 1, *A*₁), and one-half of the control culture (Text-fig. 1, *B*₁) to a fresh medium without antigen. Both experimental and control tissues underwent many passages in media with and without antigen. The remaining halves (Text-fig. 1, *A*₂ and *B*₂) of the experimental and control cultures were placed in a medium containing the antigen under a high concen-

¹ Fischer, A., *J. Exp. Med.*, 1922, xxxv, 661.

tration, from 50 to 66 per cent, which had a marked inhibiting action on the growth of normal tissues. In this way, it was possible to ascertain the resistance gained by the immunized tissue at a given time during the experiment. If the tissue grown in a medium containing a small amount of antigen constantly did not become immunized, its rate of growth was as slow as that of the control tissue



TEXT-FIG. 1. *A* is a culture with a small amount of antigen. *A*₁ and *A*₂ show the same culture divided in half. *A*₁ is carried on in a medium containing a small amount of antigen; *A*₂ (subculture) is transferred to a medium containing the antigen in a high concentration. *B* is the control culture. *B*₁ is carried on as *B*, the control culture, and *B*₂ is transferred to a medium containing the antigen in a high concentration. The quotient of *A*₂ divided by *B*₂ expresses the degree of immunization of *A*₁.

when both were exposed to a high concentration of antigen. Therefore, the quotient of the *rate of growth of the experimental subculture* (immunized strain in the high concentration of antigen) divided by the *rate of growth of the control subculture* (non-immunized strain in the high concentration of antigen) expressed the degree of immunization. For instance, if both immunized and non-immunized strains grew at

the same rate in a high concentration of antigen, the quotient was 1, and no immunization had taken place. If the immunized strain grew more actively than the non-immunized strain, the quotient was higher than 1 and expressed the degree of immunization.

The quotient of the rate of growth in their respective media of the immunized strain divided by that of the non-immunized strain expressed the effect of the small amount of antigen in which the tissues were constantly cultivated. A quotient below 1 indicated that antigen, even in a low concentration, inhibited the growth of fibroblasts. When the quotient was 1, antigen, in the concentration used, had no effect on the fibroblasts.

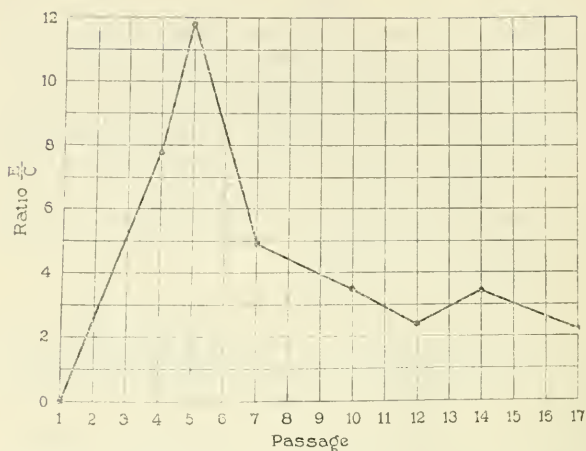
The quotient of the rates of growth of the two halves $\frac{A_1}{A_2}$ (Text-fig. 1) of the immunized strain in media containing antigen in high and low concentration expressed the effect of antigen on the immunized fibroblasts. The quotient of the rates of growth of the two halves $\frac{B_1}{B_2}$ (Text-fig. 1) of the control in media containing a high concentration and no antigen showed the effect of antigen on normal fibroblasts. These quotients were plotted in ordinates and the curves indicate that the rate of growth of the immunized strain was higher than that of the non-immunized.

II.

EXPERIMENTAL.

A. Effect of Human Ascitic Fluid as Antigen.

1. *Time and Degree of Immunization of Fibroblasts under the Influence of a Small Amount of Antigen.*—The total nitrogen of the human ascitic fluid used as an antigen amounted to 0.203 per cent. The culture medium of the immunized strain contained about 7 per cent ascitic fluid throughout the whole experiment. The results of an experiment which lasted for seventeen passages are recorded in Text-fig. 2. According to the diagram (Text-fig. 1), the ordinates in Text-fig. 2 represent $\frac{A_2}{B_2}$ (Table I), which is the rate of growth of both the immunized and non-immunized strains in high concentra-

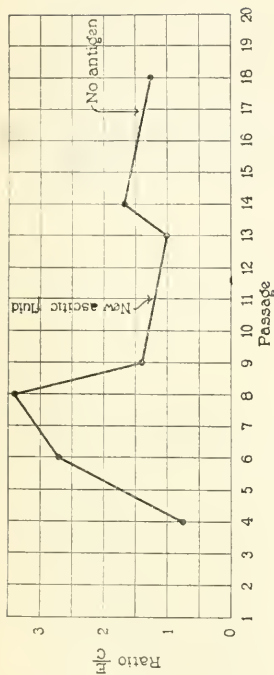


TEXT-FIG. 2. Curve showing the variations of the degree of immunization of fibroblasts through seventeen passages. Human ascitic fluid was used as antigen in a concentration of 7 per cent. The ordinates represent the quotient of the rate of growth of the immunized strain divided by that of the non-immunized strain in a high concentration of antigen, and the abscissæ the number of passages.

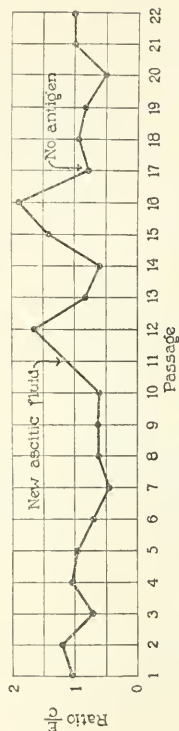
TABLE I.

Variations in the Degree of Immunization of Fibroblasts; Human Ascitic Fluid Used as Antigen in a Concentration of 7 Per Cent.

Culture No.	Passage No.	Relative increase of subcultures in 50 per cent ascitic fluid.		Ratio $\left(\frac{E}{C}\right)$ of subcultures.
		Control.	Experiment.	
847-2, 848-2	4	1.8	14.1	7.8
316-2, 317-2	5	1.1	13.0	11.8
610-2, 611-2	7	2.8	13.8	4.9
659-2, 660-2	10	4.6	16.2	3.5
686-2, 687-2	12	9.4	23.2	2.4
718-2, 719-2	14	6.0	23.0	3.4
760-2, 761-2	17	3.8	8.4	2.2



TEXT-FIG. 3. Curve showing the degree of immunization of fibroblasts through twenty-two passages. Human ascitic fluid was used as antigen in a concentration of 7 per cent. The ordinates represent the quotient of the rate of growth of the immunized strain divided by that of the non-immunized strain in a high concentration of antigen, and the abscisse the number of passages.



TEXT-FIG. 4. Curve showing the rate of growth of the immunized strain and its control through twenty-two passages. Human ascitic fluid was used as antigen in a concentration of 7 per cent. The ordinates represent the quotient of the rate of growth of the immunized strain divided by that of its control, and the abscisse the number of passages.

tions; namely, the degree of immunization. The maximum of resistance occurred at the fifth passage (10 to 12 days after the beginning of the experiment). Afterwards, the resistance declined slowly

TABLE II.

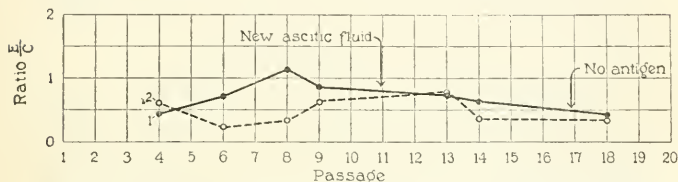
Reaction in Function of Time of Immunized and Non-Immunized Strains to a Large Amount of Antigen.

Culture No.	Passage No.	Ratio.			
		Immunized and non-immunized strains.	Non-immunized strain and its sub-culture.	Immunized strain and its sub-culture.	Subcultures of immunized and non-immunized strains.
1855-1, 1855-2	1	1.08			
1882, 1883	2	1.20			
1906, 1907	3	0.72			
1940-1, 1940-2	4	1.04	0.60	0.44	0.73
1941-1, 1941-2					
1972, 1973	5	0.98			
1993-1, 1998-2	6	0.70	0.28	0.70	2.70
1999-1, 1999-2					
2024, 2025	7	0.47			
2047-1, 2047-2	8	0.62	0.34	1.14	3.40
2048-1, 2048-2					
2073-1, 2073-2	9	0.62	0.61	0.86	1.40
2074-1, 2074-2					
2100, 2101	10	0.60			
2122, 2123	11				
2147, 2148*	12	1.63			
2170-1, 2170-2	13	0.81	0.78	0.79	1.00
2171-1, 2171-2					
2199-1, 2199-2	14	0.60	0.37	0.62	1.68
2200-1, 2200-2					
2217, 2218	15	1.46			
2238, 2239	16	1.90			
2266, 2267†	17	0.80			
2290-1, 2290-2	18	0.94	0.32	0.40	1.25
2291-1, 2291-2					
2311, 2312	19	0.84			
2337-1, 2338	20	0.53			
2363, 2364	21	1.00			
2390, 2391	22	1.10			

* In Cultures 2147 to 2239, new ascitic fluid was used.

† In Cultures 2266 to 2391, the antigen was omitted.

and persisted still after seventeen passages (about 40 days). Another experiment of the same kind was carried through twenty-two passages. Human ascitic fluid was present continuously in a concentration of about 7 per cent in the medium of the immunized strain (Text-fig. 3 and Table II). The curve (Text-fig. 3) shows the variations of the resistance of the immunized strain. The resistance quickly reached its maximum (Text-fig. 3). Afterward, it declined, but a second maximum occurred after the thirteenth passage. The curves representing this and other experiments remind one of those obtained by Jörgensen and Madsen² for the amount of agglutinin produced by a goat under the influence of continuous injections of cholera bacilli.



TEXT-FIG. 5. Curve 1 shows the variations in the quotient of the rate of growth of the immunized strain in a small amount of antigen divided by the rate of growth of the same strain in a high concentration of antigen. Curve 2 shows the variations in the quotient of the rate of growth of the non-immunized strain in the ordinary medium divided by the rate of growth of the same strain in a high concentration of antigen. Human ascitic fluid was used as antigen in a concentration of 7 per cent. The ordinates represent the quotient of the rates of growth, and the abscissæ the number of passages.

2. *Comparison of the Rates of Growth of Immunized and Non-Immunized Strains.*—The immunized strain was cultivated exactly as was the control, with the exception of the addition of 7 per cent ascitic fluid (Text-fig. 4). If the antigen at this low concentration should have any growth-inhibiting effect, the quotient of the rates of growth of the immunized strain and its control would be below 1. As the average value of the quotient was 1, it appears that the antigen did

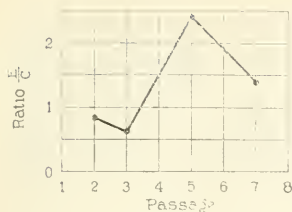
² Jörgensen, A., and Madsen, T., Festskrift ved Indvielsen af Statens Serum-Institut, Copenhagen, 1902, Paper 6, p. 12.

not markedly affect the activity of the strain. The variations in the quotient of the rates of growth of both subcultures of the immunized strain in low and high antigen concentrations are shown in Text-fig. 5, Curve 1. The quotients of the rates of growth of both subcultures of the non-immunized strain without antigen and in a high concentration of antigen are also shown in Text-fig. 5, Curve 2. These two curves indicate that the immunized strain grew more rapidly than the non-immunized. This has been the case in all the experiments. It seems that the presence of the antigen stimulated the growth of the fibroblasts during the entire experiment.

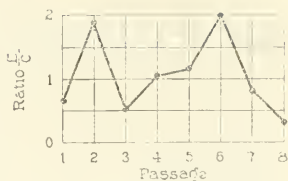
In the experiments recorded in Text-figs. 3 to 5 and Table II, new ascitic fluid was used at the eleventh passage. At the seventeenth passage, the antigen was omitted, and no change in the rate of growth occurred.

B. Effect of Dog Serum as Antigen.

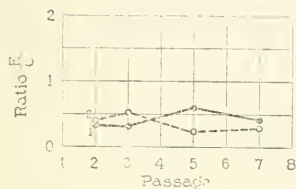
In the following experiments, dog serum was used as an antigen. When added in small quantity to the culture medium of the fibroblasts, it acted as did ascitic fluid,¹ and the fibroblasts became immunized against its action. Certain dog sera were more inhibiting for the growth of fibroblasts than others. In previous experiments,¹ the non-immunized strain of fibroblasts was often killed by a concentration of about 66 per cent, while the immunized strain remained normal. An investigation was made of the relation between the degree of immunization of a strain of fibroblasts, and the concentration of dog serum used as an antigen. Dog sera were selected which inhibited the growth of fibroblasts very markedly. The technique was similar to that previously described.¹ Fragments of a 10 year old strain of fibroblasts were immunized with antigen in different concentrations (Text-figs. 6 to 10 and Tables III to V). The antigen used was dog serum (No. 1430), in a concentration of $\frac{1}{4}$ per cent. The growth index of the subcultures $\left(\frac{A_2}{B_2}\right)$, which expresses the degree of immunity, reached a maximum at the fifth passage (Text-fig. 6). The ratio between the immunized and non-immunized strains $\left(\frac{A_1}{B_1}\right)$ shows (Text-fig. 7) that, at the seventh and eighth passages, the growth index



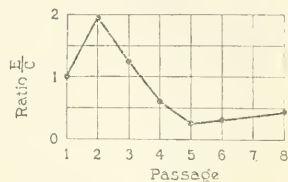
TEXT-FIG. 6.



TEXT-FIG. 7.



TEXT-FIG. 8.



TEXT-FIG. 9.

TEXT-FIG. 6. Curve showing the variations in the degree of immunization of fibroblasts through eight passages. Dog serum was used as antigen in a concentration of 4 per cent. The ordinates represent the quotient of the rate of growth of the immunized strain divided by that of the non-immunized strain in a high concentration of antigen, and the abscissæ the number of passages.

TEXT-FIG. 7. Curve showing the action of antigen on the immunized strain through eight passages. Dog serum was used as antigen in a concentration of 4 per cent. The ordinates represent the quotient of the rate of growth of the immunized strain divided by the rate of growth of its control, and the abscissæ the number of passages.

TEXT-FIG. 8. Curve 1 shows the variations in the quotient of the rate of growth of the immunized strain in a small amount of antigen divided by the rate of growth of the same strain in a high concentration. Curve 2 shows the variations in the quotient of the rate of growth of the non-immunized strain in the ordinary medium divided by the rate of growth of the same strain in a high concentration of antigen. Dog serum was used as antigen in a concentration of 4 per cent. The ordinates represent the quotient of the rates of growth, and the abscissæ the number of passages.

TEXT-FIG. 9. Curve showing the variations in the degree of immunization of fibroblasts through eight passages. Dog serum was used as antigen in a concentration of 8 per cent. The ordinates represent the quotient of the rate of growth of the immunized strain divided by the rate of growth of the non-immunized strain in a high concentration of antigen, and the abscissæ the number of passages.

TABLE III.

Immunization of Fibroblasts by Dog Serum in 4 Per Cent Concentration of Antigen.

Culture No.	Passage No.	Ratio.			
		Immunized and non-immunized strains.	Non-immunized strain and its sub-culture.	Immunized strain and its sub-culture.	Subcultures of immunized and non-immunized strains.
1180, 1181	1	0.68			
1216-1, 1216-2	2	1.90	0.43	0.36	0.86
1217-1, 1217-2					
1255-1, 1255-2	3	0.51	0.50	0.30	0.60
1256-1, 1256-2					
1289, 1290	4	1.03			
1326-1, 1326-2	5	1.17	0.25	0.59	2.40
1327-1, 1327-2					
1361, 1362	6	2.00			
1397-1, 1397-2	7	0.80	0.30	0.41	1.40
1398-1, 1398-2					
1452, 1453	8	0.30			

TABLE IV.

Immunization of Fibroblasts by Dog Serum in 8 Per Cent Concentration of Antigen.

Culture No.	Passage No.	Ratio of subcultures of immunized and non-immunized strains.
1491-2, 1492-2	2	1.95
1713-2, 1714-2	3	1.25
1489-2, 1490-2	4	0.60
1538-2, 1539-2	5	0.25
1094-2, 1095-2	6	0.30
1170-2, 1171-2	8	0.45

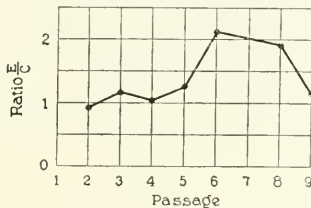
TABLE V.

Immunization of Fibroblasts by Dog Serum in 1.4 Per Cent Concentration of Antigen.

Culture No.	Passage No.	Ratio of subcultures of immunized and non-immunized strains.
1118-2, 1119-2	2	0.92
1257-2, 1258-2	3	1.14
1602-2, 1603-2	4	1.06
1670-2, 1671-2	5	1.23
1637-2, 1638-2	6	2.13
1456-2, 1457-2	8	1.90
1908-2, 1909-2	9	1.17

being under 1, the growth of the immunized strain was very much inhibited. It indicates also that after a few passages (Text-fig. 8) the immunized strain grew more actively than the non-immunized strain.

When the antigen (Dog Serum 1430) was used in a concentration of about 8 per cent, the maximum of resistance was reached at the second passage and lasted only a short time (Text-fig. 9 and Table IV). Afterwards, the resistance fell rapidly below 1. This experiment demonstrated also that, in spite of the marked inhibiting effect of an 8 per cent concentration of antigen, the strain presented a much higher resistance against dog serum than the control immediately after the second passage. When 1.4 per cent antigen was added to



TEXT-FIG. 10. Curve showing the variations in the degree of immunization of fibroblasts through nine passages. Dog serum was used as antigen in a concentration of 1.4 per cent. The ordinates represent the quotient of the rate of growth of the immunized strain divided by the rate of growth of the non-immunized strain in a high concentration of antigen, and the abscissæ the number of passages.

the culture medium, a different result was obtained (Text-fig. 10 and Table V). This low concentration of antigen restrained the growth of the immunized strain, but was less toxic for the fibroblasts than a 5 to 8 per cent concentration. The resistance reached its maximum at the sixth or eighth passage and decreased slowly afterwards.

III.

SUMMARY AND CONCLUSIONS.

The action of human ascitic fluid and of dog serum on the immunization of fibroblasts *in vitro* was investigated. The toxic effect of ascitic fluid upon fibroblasts was slight, while that of dog serum was

marked. When a small amount of antigen (1.5 to 2 per cent dog serum, or 8 per cent ascitic fluid) was present in the medium, the resistance of the fibroblasts slowly increased, reached its maximum after about 14 days, and gradually decreased. The curve expressing the phenomena closely resembles that of Jörgensen and Madsen showing the production of antibodies in an animal which received daily injections of antigen. When the antigen was present in the medium in a high concentration (from 5 to 8 per cent dog serum), the resistance reached its maximum in 4 days, and decreased rapidly.

It may be concluded that:

1. In the immunization of fibroblasts *in vitro* against a foreign protein, there is a relation between the amount of antigen, the time of the appearance of immunization, and its duration.
2. When a small amount of antigen is used, immunization slowly reaches its maximum, and slowly decreases.
3. When a large amount of antigen is used, immunization reaches its maximum in a short time, but the protection is of equally short duration.

I wish to acknowledge my indebtedness to Dr. Alexis Carrel for his permission to use the old strain of fibroblasts. I also wish to express my gratitude for the help and kindness of Dr. Carrel and his associates during my stay at The Rockefeller Institute.

SURFACE TENSION OF SERUM.

IV. ACTION OF TEMPERATURE.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 50 TO 53.

(Received for publication, May 25, 1922.)

The action of heat on the surface tension of serum has been reported in previous papers,^{1,2} but only at a temperature of 55°C., and for a short time. The purpose of this paper is to show the effect of prolonged heat at 55°C. on the initial value of the surface tension of pure serum, and that of temperatures of 55°, 70°, and 100°C. on the time-drop of serum solutions. In order to save space, it is assumed that the reader is familiar with the technique and terms described and used in the preceding articles.

I.

Pure Serum.

In the case of pure serum, only the initial value of the surface tension was measured, because the changes in the time-drop would be almost of the order of magnitude allowed for experimental errors. Dog serum was kept in an incubator at 55°C. for 168 hours (7 days). Measurements were made every 24 hours, according to the technique referred to above. The results are shown in Tables I to IV and Text-figs. 1 to 3.

These figures show plainly the continuous decrease of the value of the surface tension when pure serum is heated continuously. The smoothness of the curves indicates that, in most cases, the modifications undergone by the serum between the 1st and 24th hours, and between the 24th and 48th hours, for instance, are quite similar.

¹ du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 575, 707.

² du Noüy, P. L., *J. Exp. Med.*, 1922, xxxvi, 115.

Consequently, the destruction of complement which takes place in the first 2 hours does not manifest itself by an important change in the value of the surface tension, or else the curves would show some kind of a break, a maximum or a minimum; moreover, if the change is

TABLE I.

Dog Serum, No. 976 (Text-Fig. 1).
Temperature 23°C.

Time, hrs.....	0	24	48	72	96	120	130
Surface tension, dynes.....	57.0	56.5	56.0	55.0	54.0	53.0	Coagulated.

TABLE II.

Dog Serum, No. 984 (Text-Fig. 1).
Temperature 23°C.

Time, hrs.....	0	24	48	72	96	120	130
Surface tension, dynes.....	56.0	55.0	53.0	51.9	52.5	50.0	Coagulated.

TABLE III.

Dog Serum, No. 998 (Text-Fig. 2).
Temperature 23°C.

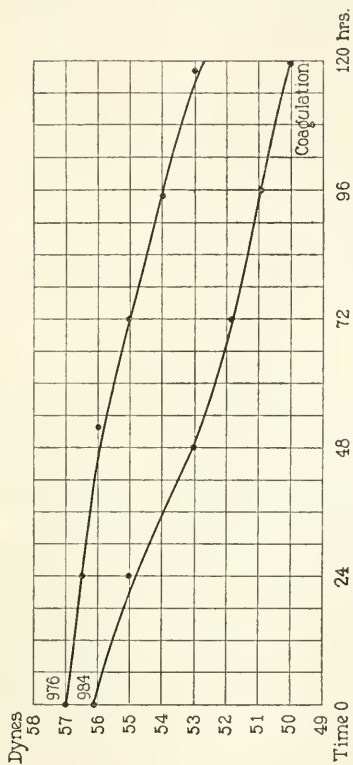
Time, hrs.....	0	24	48	72	96
Surface tension, dynes.....	57.0	57.0	56.0	55.0	54.1

TABLE IV.

Dog Serum, No. 987 (Text-Fig. 3).
Temperature 23°C.

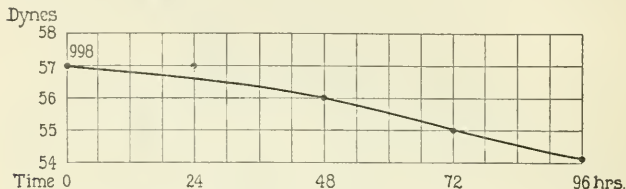
Time, hrs.....	0	24	48	72	96	120	130	168
Surface tension, dynes.....	57.5	54.5	52.3	51.3	50.8	50.5	50.0	49.2

connected in any way with the decrease in surface tension, it is so small in 2 hours that it remains within the range of experimental error. At the end of 120 hours, the reaction is in general almost as marked as at the beginning, the slope of the curve showing no constant tendency to change.



TEXT-FIG. 1. Action of heat at 55-56°C. on the surface tension of serum, in function of the time.

As the perturbations due to the destruction of complement may be of such order of magnitude that the presence of highly concentrated colloids would hinder their effect on surface tension, if there is any, experiments were carried out with solutions of serum.



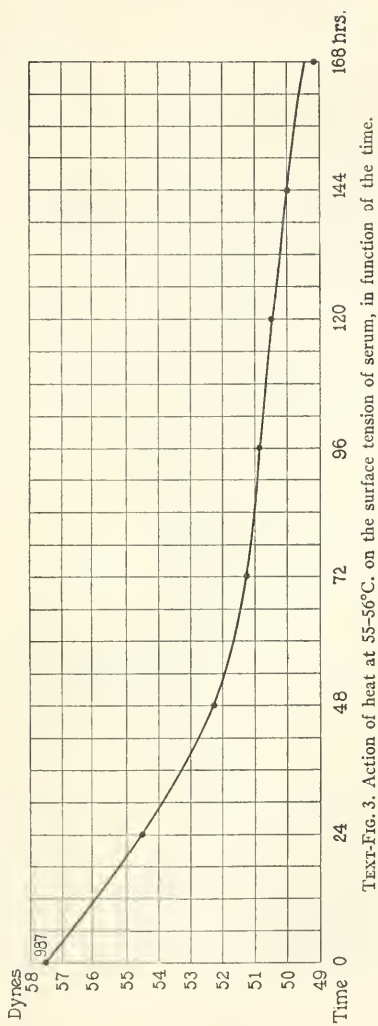
TEXT-FIG. 2. Action of heat at 55-56°C. on the surface tension of serum, in function of the time.

II.

Solutions of Serum.

All solutions were made from the same serum, with 0.9 per cent NaCl solution, especially prepared in clean vessels, the surface tension of which was $76.0 \text{ dynes} \pm 0.2$ at 23°C. The solutions were stirred mechanically by an electric motor fitted with a small, propeller-shaped, glass stirrer. The liquid was removed from the container while the stirring was going on and poured into clean test-tubes.

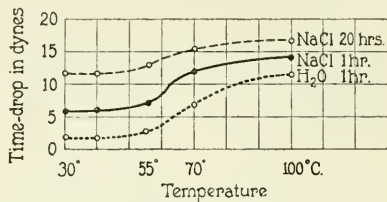
The heating was done in the following way. In most experiments six dilutions were prepared, 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} . About 6 cc. of each dilution were poured into four test-tubes which were prepared at the same time. One was kept at room temperature, one at 55°C. for 2 hours, one at 70°C. for 1 hour, and one at 100°C. for 5 minutes. In order that the same time should elapse between the preparation of the solutions and the measurements, the measurements were begun only 1 hour after the tube at 55°C. had been removed from the incubator, so that approximately 3 hours elapsed between the pouring into the test-tubes and the measurements. Before the liquid was poured into the watch-glasses, it was carefully stirred. Then after 2 hours, another reading was taken. Hence the time required for one set of measurements, at one concentration, was 5 hours. In this case, the time-drop was studied because the initial values, on ac-



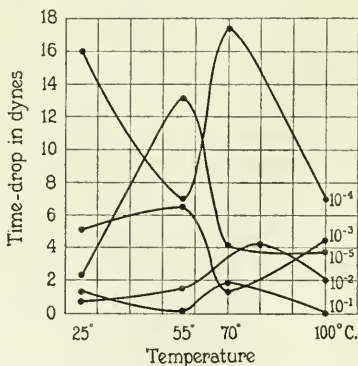
TEXT-FIG. 3. Action of heat at 55-56°C. on the surface tension of serum, in function of the time.

count of the dilution, do not show any marked differences, except when heated at 100°C .

In order to save space, and because the accuracy of the figures does not have any bearing on the general results, charts only will be

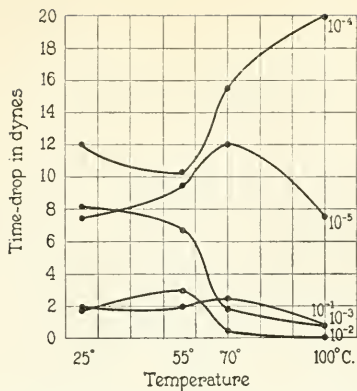


TEXT-FIG. 4. Time-drop of serum solution, concentration 10^{-4} , in saline solution and distilled water (Serum 119).

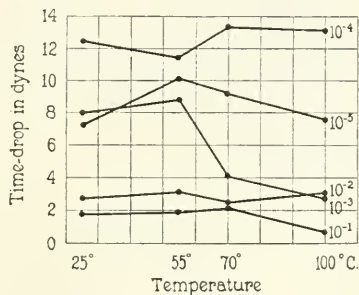


TEXT-FIG. 5. Time-drop in 2 hours, in function of the temperature, at different concentrations (Serum 1696).

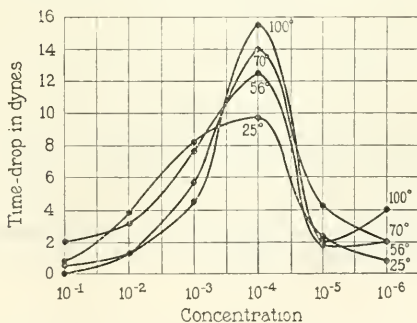
given. A curve expressing the time-drop in 1 and in 20 hours at one concentration is given in Text-fig. 4, with the curve obtained by diluting the serum in distilled water. A fair idea of how puzzling the results seem to be at first glance is given in Text-figs. 5 to 10. No regularity whatsoever is to be found. Unless an hypothesis is made, it is im-



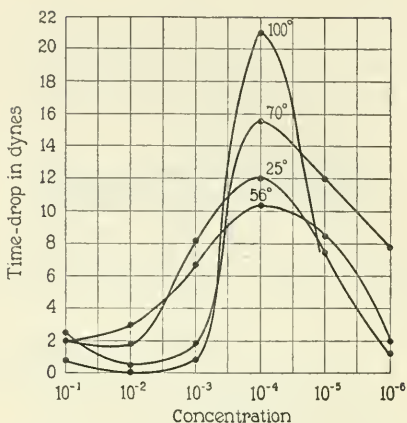
TEXT-FIG. 6. Time-drop in 2 hours, in function of the temperature, at different concentrations (Serum 1711).



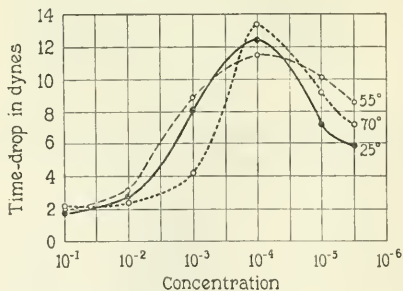
TEXT-FIG. 7. Time-drop in 2 hours, in function of the temperature, at different concentrations. Mean values of ten experiments.



TEXT-FIG. 8. Time-drop in 2 hours, in function of the concentration, at different temperatures (Serum 1802).



TEXT-FIG. 9. Time-drop in 2 hours, in function of the concentration, at different temperatures (Serum 1711).



TEXT-FIG. 10. Time-drop in 2 hours, in function of the concentration, at different temperatures. Mean values of ten experiments.

possible to interpret these results. The mean values of ten of the charts, in other words the algebraic sums of 480 measurements, are represented by Text-figs. 7 and 10.

III.

DISCUSSION.

The interpretation of these results is difficult, and it is not at all certain that it will be successful. However, we shall endeavor to give a possible explanation of the curves, which seem, at first sight, contradictory and devoid of meaning.

One is struck by the fact that the most important changes in the time-drop occur at the temperatures 55° and 70°C. (Text-figs. 5 to 7). These temperatures seem to be critical for the serum, and the fact should have attracted attention even had it not been known that they are also critical from the physiological standpoint. On the other hand, the curves representing the behavior of solutions 10^{-1} and 10^{-2} are parallel in general, and do not present the sharp jumps and drops shown by solutions 10^{-3} , 10^{-4} , and 10^{-5} . Finally, it will be noticed that the dilutions 10^{-3} and 10^{-4} generally act in an opposite way.

The first idea which naturally arises is that the surface equilibrium is more stable on either side of the concentration 10^{-4} , that is 10^{-3} and 10^{-5} , so that around 10^{-4} it reaches some sort of peak instability, and the values may rise or drop suddenly, according to the laws of hazard; namely, for such reasons as actually escape our control and knowledge. These causes, although very slight, may produce considerable changes in either direction simply because, the equilibrium being unstable, the phenomenon has just as much tendency to occur one way as the other.

Table V shows, perhaps more clearly than the charts, that the surface equilibrium at the concentration 10^{-4} seems perfectly unstable, while it is more or less determined in a given direction at all other concentrations. Here, the direction only of the time-drop has been taken into account, whether it increased or decreased, at the critical temperatures. The + sign expresses symbolically that the time-drop rose, the - sign that it decreased. As the values were taken from ten experiments, the coefficients were added to show the percentage of the occurrence of the rise or the drop; and it was admitted that one - sign checks a + sign, so that the figures actually express the number of rises or falls in excess. For example, out of the 10 experiments at a dilution 10^{-1} between 25° and 55°C., 2 showed a decrease and

8 showed an increase of the time-drop. The 2 decreases will be given the $-$ sign, and the 8 increases the $+$ sign. But 2 $-$ counteracts 2 $+$, so that we have only 6 $+$ left. The next entry shows that we had 9 $+$ and 1 $-$, the result being 8 $+$. In other words, the figures could be called equilibrium coefficients, 10 being the maximum characterizing perfect equilibrium, and 0 (5 $+$ and 5 $-$) corresponding to perfect instability.

Table V shows that there is a decided maximum of instability at the concentration 10^{-4} , and a much greater instability on the side of higher dilutions than on the side of lower dilutions. As a matter of fact, the presence of the 3 zeros in the 10^{-4} line may be considered as an accident, on account of the small number of experiments. But

TABLE V.

Increase and Decrease of the Time-Drop of Serum Solutions in 2 Hours When Heated at 55°, 70°, and 100°C.

Concentration,	Between 25° and 55°C.	Between 25° and 70°C.	Between 55° and 70°C.	Between 70° and 100°C.
10^{-1}	6+	10+	8+	10-
10^{-2}	8+	2-	8-	0
10^{-3}	6+	10-	10-	2-
10^{-4}	0	0	0	2+
10^{-5}	2+	2+	0	2-
10^{-6}	2-	2-	10-	2+

the results would have been just as convincing had such coefficients as 2 been found; only 100 experiments would be expected to give a real expression of the state of instability. However, there is no doubt that there is a striking difference, which could only be enhanced by a larger number of data, between the upper part of the table and the lower.

This confirms our preceding hypothesis as to the existence of a monomolecular layer at a concentration around 10^{-4} , geometrically arranged so that the sum of all individual molecular attractions in this layer is minimum, or very nearly so. Hence, any minute change, either in the arrangement of the molecules or else in their field of force, will manifest itself by a relatively considerable rise or decrease in the time-drop, owing to the state of unstable equilibrium of the

film. Therefore, it is in the vicinity of this concentration that the most sudden jumps of the surface tension are to be expected (Text-figs. 8 and 9).

From these conclusions, one fact may be remembered; namely, that if certain physiological modifications of the serum are to have a bearing on surface tension or its changes in function of the time, the possibility of detecting them will be greater at the dilution which enables one to bring forth the slightest alterations in the fields of forces of the molecules or group molecules, that is around 1:10,000.

IV.

Crystallization.

Figs. 1 to 4 show the alterations brought about by heating the solution of serum at 1:10, in the crystallization of sodium chloride. However, the changes are not always identical, especially between 25° and 56°C., and all sera do not allow the sodium chloride to crystallize in the same way. The pictures reproduced here are, nevertheless, frequently observed, and other configurations may be considered as more or less exceptional. The reader is referred to the preceding paper² for another manifestation of the action of heat on solutions of serum.

V.

CONCLUSIONS.

1. The surface tension of pure serum, heated at 55°C., decreases progressively and regularly until the serum coagulates. A drop of 8.2 dynes was observed in 168 hours. The mean drop was 5.7 dynes in 120 hours and 4 dynes in 96 hours.

2. The initial surface tension of solutions of serum at the concentrations 10^{-1} up to 10^{-6} is practically not affected by heat, but the time-drop in 2 hours is modified.

3. Each serum seems to react in its own particular way as far as the time-drop is concerned. However, there is a general tendency for the solution to show an increase of time-drop at the concentrations 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-5} , and a decrease at 10^{-4} , when heated at 55°C.; a decrease of the time-drop at the concentrations 10^{-1} , 10^{-2} , and 10^{-3} , and an increase at 10^{-4} when heated at 100°C.

4. Nevertheless, only the changes observed below 10^{-3} are constant in sign, within 75 or 80 per cent of the cases. The concentration 10^{-4} seems to correspond to a state of greater instability. This confirms the hypothesis of the existence of a monomolecular layer at that concentration, which was assumed on the basis of the existence of a maximum drop at 10^{-4} . Should this be true, and provided the principle of Gibbs could be transformed so as to be applicable to mixed solutions of colloids and crystalloids, an idea of the size, or at least of one of the dimensions of the molecules or group molecules composing the serum could probably be obtained.

5. 1 drop of the solutions at 1:10 evaporated on glass and examined under the microscope shows the marked differences brought about by heating at different temperatures.

A great number of the measurements referred to in this paper were made by my technical assistant, Mr. John Zwick, to whom I wish to express my indebtedness.

EXPLANATION OF PLATES.

PLATE 50.

FIG. 1. Photomicrograph of 1 drop of serum diluted in 1:10 saline solution. Kept at room temperature. $\times 20$.

PLATE 51.

FIG. 2. Photomicrograph of 1 drop of serum diluted in 1:10 saline solution. Kept at 55°C. for 2 hours. $\times 20$.

PLATE 52.

FIG. 3. Photomicrograph of 1 drop of serum diluted in 1:10 saline solution. Kept at 70°C. for 1 hour. $\times 20$.

PLATE 53.

FIG. 4. Photomicrograph of 1 drop of serum diluted in 1:10 saline solution. Kept at 100°C. for 5 minutes. $\times 20$.



FIG. 1.

(du Noüy: Surface tension of serum. IV.)



FIG. 2.

(du Noüy: Surface tension of serum. IV.)

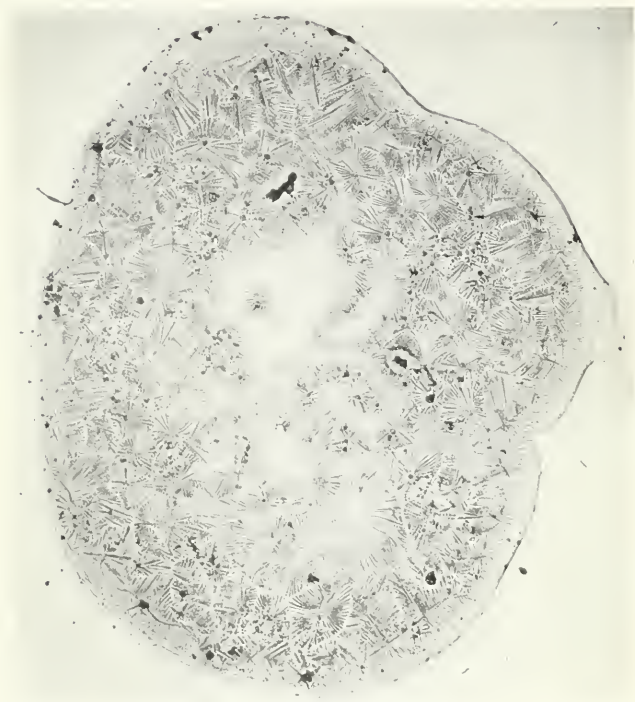


FIG. 3.

(du Noüy: Surface tension of serum. IV.)

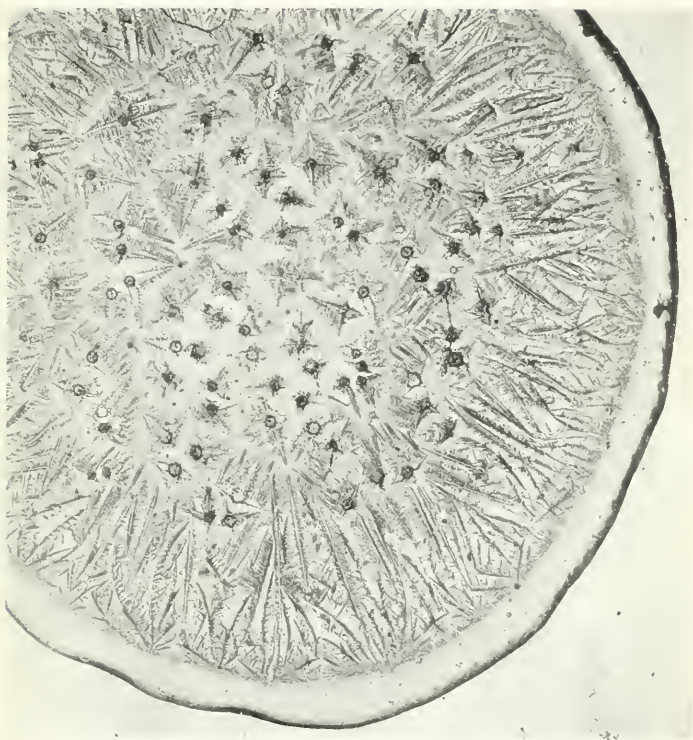


FIG. 4.

(du Noüy: Surface tension of serum. IV.)

INVESTIGATIONS ON THE OXYGEN CONTENT OF CUTANEOUS BLOOD (SO CALLED CAPILLARY BLOOD).

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(Received for publication, June 16, 1922.)

INTRODUCTION.

It is generally presumed that cutaneous blood (so called capillary blood) is similar to arterial blood, particularly if the skin is made hyperemic by rubbing, or application of heat, etc., before the incision is made (Naegeli (1)). That cutaneous blood, at any rate so far as blood sugar is concerned, differs from the venous blood, has been shown by Hagedorn (2), who also uses the hypothesis that the capillary blood is similar to arterial blood in that respect. In the following experiments we have taken up the question of the composition of the cutaneous blood and especially its relation to the arterial blood. We have used the oxygen content as an indicator of the nature of the blood, first, because the amount of oxygen in the blood generally changes during the blood flow through the capillaries, and second, because oxygen unlike all other substances in the blood has the property¹ that its maximum amount in the blood can be determined outside the body, this being equal to the sum of the total oxygen capacity of the hemoglobin and the amount dissolved in the plasma. If no appreciable change takes place in the concentration of the hemoglobin when the blood flows from the arteries to the cutaneous incision, the oxygen content of the arterial blood must lie between the total oxygen capacity and the content of the cutaneous blood. It is *a priori* probable that this range is small and that for many purposes cutaneous blood could be substituted for blood obtained by

¹ This property is a consequence of the form of the oxyhemoglobin dissociation curve.

7	5	Cutaneous (1), (121)	(22.36)	21.90	0.46	97.9	+	Patient, age 40, with emphysema, bronchial asthma, and chronic bronchitis. No attack of asthma in last 24 hrs. Several rhonchi, a few coarse râles heard at back of both lungs. No fine râles. Heart normal. The two first samples taken after rest and immediately after incision; last two samples taken after patient had walked twice up and down one flight of stairs. No cyanosis present.
		Venous (1), (121)	22.36	18.52	3.84	82.9	-	
		Cutaneous (2), (126)	23.38	20.82	2.56	89.1		
		Venous (2), (126)	(23.38)	19.47	3.91	83.3		
8	1	Cutaneous (1), (101)	18.71	11.81	6.90	63.1	+	Normal individual at rest. Two first samples (cutaneous blood 1 and venous blood) taken when subject inspired air with very low oxygen tension (42.77 mm.). Very heavy generalized cyanosis was observed at that time. Blood drawn immediately after incision. The third sample (cutaneous blood 2) was taken shortly after the first and from the same incision, but at a period during which the subject inspired normal air. No cyanosis was present at that time.
		Venous, (117)	21.64	10.50	10.50	51.5	-	
		Cutaneous (2), (100)	18.52	17.62	0.90	95.14		

*The figures in parentheses are the calculated figures.

arterial puncture. The object of these experiments was to show whether this is actually the case.

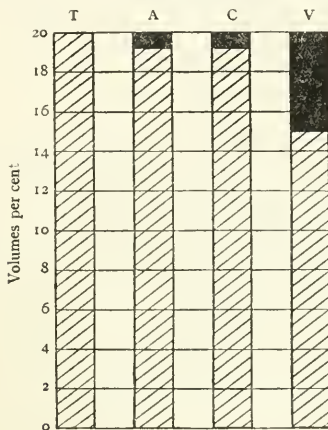
Technique.

1. *Drawing the Blood.*—Arterial puncture, which was made in only one instance (Experiment 1), was performed according to the technique of Hürter (3), Stadie (4), and Harrop (5). Venous puncture was done according to the procedure devised by one of us (6). For collecting the cutaneous blood the following method was devised. With a very sharp knife, from one to three incisions were made in the finger-tip, in some instances without, and in some instances with previous freezing with ethyl chloride (Table I). The incisions were from 1 to 1.5 cm. long, and approximately 3 mm. deep and were made lengthwise on the finger-tip. After the incision was made and the first drop of blood taken away, the subject himself compressed the wound with the thumb of the same hand and the two fingers were put down under oil in a jar. Pressure with the thumb was then discontinued, the blood thus being allowed to run freely under the layer of paraffin. A small amount of pulverized potassium oxalate was put in the bottom of the jar in order to prevent coagulation of the blood. During the bleeding the blood was stirred with an absolutely smooth glass rod. This was done very gently in order not to mix the blood with drops of paraffin. As a rule 3 to 4 cc. of blood were taken during a period of from 1 to 2 minutes.

2. *Determination of the Oxygen Content of the Blood.*—Van Slyke's method was employed. For each analysis 1 cc. of blood was used, and in all cases duplicate determinations were made. In one instance the Krogh modification of the Haldane-Barcroft method (differential method) was used because the amount of blood taken from the cutaneous incision was too small to allow duplicate determinations by Van Slyke's method. The total oxygen capacity was determined either by using a standardized Autenrieth colorimeter, or by saturating a sample of blood and determining the amount of oxygen. For this procedure only 1 cc. of blood was necessary because the blood was saturated within the Van Slyke apparatus as previously described (7). In Table I are given the figures for the hemoglobin value and for the total oxygen capacity.

EXPERIMENTS.

Experiment 1. Normal Resting Individual.—After the subject had rested on a bed for 5 minutes, a puncture of the left radial artery was made.² The moment that blood appeared in the syringe, blood was drawn from the right vena mediana cubiti. An incision was made in the tip of the second finger of the right hand without any previous freezing. 20 cc. of arterial blood were taken during a period of 5 seconds, 12 cc. of venous blood during 10 seconds, and 2 cc. of cutaneous blood during 45 seconds.³ The results of the analyses are given in Table I and in Text-



TEXT-FIG. 1. Diagram showing the total oxygen-combining power of the blood (*T*), the oxygen content of the arterial blood (*A*), the oxygen content of the sample of blood obtained from an incision in the finger-tip (*C*), and the oxygen content of the blood from the vena mediana cubiti (*V*). All are given in volumes per cent (cubic centimeters of oxygen per 100 cc. of blood) and refer to Experiment 1 in Table I. The black part of the column indicates the amount of reduced hemoglobin, and the cross-hatching the amount of oxyhemoglobin. The total oxygen-combining power of the blood in this patient was 20, corresponding to a hemoglobin percentage of 108.

² The arterial puncture was done by Dr. George A. Harrop, Jr., of Baltimore.

³ Of the five subjects, two (Nos. 1 and 2) are the authors. One (No. 4) was a private patient who volunteered. The other two were patients in the University Clinic (No. 3 was one of the nurses) who were willing to cooperate.

fig. 1. Both arterial and cutaneous blood were almost fully saturated with oxygen. The former showed 97.5 per cent and the latter 96.6 per cent of the total amount (20 volumes per cent) which the blood could absorb according to its hemoglobin content. The venous blood was 75 per cent saturated.

The percentage saturation of the arterial blood was in accordance with the results of previous investigators (Hürter (3), Barcroft (8), Stadie (4), Harrop (5), and Meakins and Davies (9)), who showed that in normal individuals arterial blood is from 90 to 100 per cent saturated. The value for venous saturation, incidentally, agreed with that previously found by one of us as the average saturation of venous blood in normal resting individuals (6). For cutaneous blood no previous determinations have been made.

Experiment 2. Normal Resting Individual.—After 15 minutes rest on a bed, an incision was made in the tip of the fourth finger of the left hand after previous freezing. During 2 to 3 minutes 4 to 5 cc. of blood were collected. During that period a sample of blood was drawn from the left vena mediana cubiti. Analysis of the two blood samples yielded results which were in accordance with those of the first resting subject. The cutaneous blood was almost completely saturated with oxygen (98 per cent), whereas the saturation of the venous blood was 65.8 per cent. The total oxygen capacity of the blood was found by direct determination to be 18.79 volumes per cent. No arterial puncture was made. We know that the saturation of the arterial blood in this case will be between 100 per cent, which is the total oxygen-combining power of the blood, and 98 per cent, which is the degree of saturation of the cutaneous blood. From the dissociation curve of the oxygen hemoglobin, and from the analyses of the arterial blood made by Hürter, Barcroft, Stadie, Harrop, and Meakins and Davies, we know that the saturation of the arterial blood is more likely to be 98 than 100 per cent. Therefore in this case too the cutaneous blood can be considered identical with the arterial blood as far as oxygen content is concerned.

Experiment 3. Normal Individual after Exercise.—After three unsuccessful attempts by Dr. Harrop to do arterial puncture, the subject before each attempt having run very quickly five times up and down a flight of stairs, the arterial puncture was given up. The subject again ran very quickly five times up and down a flight of stairs, and immediately after this an incision was made in the tip of the third finger of the right hand without previous freezing. 3 cc. of blood were taken during a period of 1 minute. Simultaneously a sample of venous blood was drawn from the arm of the same subject. The oxygen content of the cutaneous blood was found to be a trifle lower than that found in the previous experiments on resting individuals, but was within the normal limits of the oxygen content of arterial blood. The figure (94.3 per cent) approximates very closely that usually given as the average figure for normal resting individuals. The oxygen content of the

venous blood was the same as that usually found in normal resting individuals, but it differs from the values found in a series of experiments on the oxygen content of the venous blood after exercise in similar individuals (10). The analyses show that the largest possible discrepancy that can exist between the oxygen saturation of arterial and cutaneous blood is 5.7 per cent. The difference between the two figures is undoubtedly much smaller.

Experiment 4. Normal Individual after Exercise.—An incision was made in the tip of the fourth finger of the left hand after previous freezing. The finger was bandaged with gauze soaked in paraffin. After this the subject ran five times up and down a flight of stairs very quickly, taking 1 minute and 15 seconds. After the exercise his pulse was 132 and his respiration 40. 3 cc. of blood were taken during a period of 1 minute in the usual way from the incision already made. The cutaneous blood was in this instance also almost totally saturated (98.2 per cent). The saturation of the arterial blood must, of course, be between the total oxygen capacity, which was 22.18 volumes per cent, and the oxygen content of the cutaneous blood, which was 21.78 volumes per cent.

Experiment 5. Patient with a Large Exudate in the Left Pleura; an Almost Total Compression of the Left Lung.—A clinical and x-ray examination showed that the left lung was practically compressed. The patient was rather pale. No cyanosis was present. Pulse was 132 and respiration 58. Without previous freezing, two incisions, each 1 cm. long, were made in the tip of the fourth finger of the right hand, and simultaneously an incision was made in the right vena mediana cubiti. The saturation of the cutaneous blood was 93.2 per cent, which was somewhat lower than the average for normal resting individuals, but still within normal limits. The saturation of the venous blood was normal (72.7 per cent). The arterial blood must in this case have been saturated to at least 93.2 per cent. These figures and the result of the x-ray and clinical examinations make it certain that the left lung must have been compressed to such an extent that not only was the passage of air prevented, but also the blood flow. This furthermore is in accordance with the fact that the patient was not cyanotic (10).

Experiment 6. Patient with Compensated Mitral Stenosis, before and after Exercise.—After 30 minutes of absolute rest, an incision was made in the tip of the fourth finger of the left hand, the finger having previously been frozen. 4 cc. of blood were taken from the incision during a period of 2 minutes. At the same time a puncture was made in the left vena mediana cubiti. The pulse rate was 100 and the respiration 24. Then the finger was bandaged and the patient went slowly up stairs, one flight, and was put to bed. Immediately after this another sample of venous blood was taken from the same vein as before. An attempt was made to get a sample of cutaneous blood but without success on account of coagulation of the blood in the incision. Just after the patient had climbed the stairs the pulse rate was 132 and the respiration 36. During rest her lips were cyanotic, whereas the color of her finger-tips was more pinkish (erythrosed). After exercise the cyanosis was somewhat increased without becoming general. Her finger-tips were somewhat cyanotic. The analyses showed

that the cutaneous blood taken during rest was almost fully saturated with oxygen (98.2 per cent). We can therefore consider the cutaneous blood in this case to be identical with the arterial blood as far as the oxygen content is concerned. The experiment also shows that if any part of the lungs were not sufficiently ventilated (a few coarse râles were heard in the back of both sides) these parts could not have been passed by any blood, because had that occurred, the result would have been an abnormally high unsaturation of arterial and cutaneous blood. This, as the analyses show, was not found to be the case. Analyses of the two samples of venous blood taken before and after exercise yielded quite different results. At rest the venous blood was found to be 69.3 per cent saturated, which corresponds fairly well to the saturation of the average normal resting individual. This is also in accordance with the fact that no cyanosis of the fingers was present at that time. As already mentioned, however, the lips were cyanosed while the subject was resting. This cyanosis must necessarily be of peripheral (capillary, non-pulmonary) nature, and mainly due to deoxidation within the capillaries in the lips, because the arterial blood as shown was at least 98.2 per cent saturated. After the exercise, which was very slight, the venous blood contained only a small amount of oxygen, being 29.4 per cent saturated. This figure gives a venous unsaturation of 14.4 volumes per cent. At that time a slight cyanosis of the fingertips was observed. There seem to be two possible explanations of the heavy increase in the oxygen unsaturation of the venous blood after exercise: one, that it was due to insufficient oxidation of the blood in the lungs, the result of which would be increased unsaturation of the arterial and capillary blood; the other, that an increased deoxidation in the periphery had taken place. The latter explanation seems to be the more likely on account of the fact that no general cyanosis was observed after exercise. From previous investigations we know that cyanosis of pulmonary origin is usually more or less generalized; but in the case of this patient the cyanosis was not general. While she was climbing the stairs her hands and arms were kept perfectly quiet so that exercise could not have directly produced an increased deoxidation in the hands on account of increased local metabolism in the arm. If her cyanosis was of a peripheral nature and due to increased deoxidation in the capillaries of the hands, this deoxidation must have been due to a rather marked vascular constriction causing a decrease in the local blood flow. We have, in another series of experiments on normal individuals, observed a similar condition after heavy exercise (11, 12) (running quickly five times up and down one flight of stairs), and we have brought out different facts which seem to show that this increased oxygen unsaturation of the venous blood was due to slow local blood flow owing to vascular constriction in the arms which were at rest during the experiment. The vasoconstriction would tend to increase the blood flow in the active parts (lower extremities) during exercise. If the same explanation applies to this patient, it would show that a very slight amount of exercise brought her vasoconstrictory mechanism in the resting regions into activity. With a decrease in the blood flow in the resting parts the patient could perform the exercise with a smaller increase in the minute volume of the heart than would otherwise have been the case.

Experiment 7. Patient with Pulmonary Emphysema, Bronchial Asthma, and Chronic Bronchitis, before and after Exercise.—The patient had a chest of typical emphysematous shape. No absolute heart dullness was present, but apart from that the examination of the heart was normal. The lungs extended on the back to the twelfth rib, and on the right in front to the seventh rib. In both lungs a number of rhonchi and a few coarse râles were observed. No fine râles were present. The patient was not cyanotic. During rest the pulse was 96, and the respiration 24. After exercise the respiration went up to 32. The pulse rate was not observed. After 15 minutes rest on a bed an incision was made in the finger-tip after previous freezing. A sample of venous blood was taken at the same time. The patient then walked twice quickly up and down one flight of stairs. Immediately after exercise and without previous freezing, an incision was made in another finger of the same hand as before. Simultaneously a sample of venous blood was drawn from the same vein. The analyses of the blood taken during rest showed the same results that are found in normal resting individuals, the cutaneous blood being almost fully saturated with oxygen (97.9 per cent). Therefore, in this case it appears, first, that we are justified in considering the arterial and cutaneous blood as identical, at any rate as far as the oxygen content is concerned; and second, that the lungs, in spite of their pathological condition, allowed the blood to be normally saturated during its passage through the pulmonary circulation. This is in accordance with the results of M. Krogh's (13) determinations of the diffusion constant for the lungs in four resting emphysematous patients. In four instances this author's figures were within the limits which she previously had established as normal. After a rather slight amount of exercise, an oxygen unsaturation in the cutaneous blood of 2.56 volumes per cent was observed. This corresponded to a saturation of 89 per cent, a figure which is just below the lower limit of the oxygen saturation in the arterial blood in normal resting individuals. Determination of the saturation of arterial blood in normal individuals during exercise has been made by Harrop (5) and Barcroft and his collaborators (14). In both studies a slight decrease in the arterial oxygen saturation was found after very heavy exercise. If the cutaneous blood in our patient, also after exercise, can be considered identical with the arterial blood, our experiment might indicate that the patient's lungs were not quite capable of meeting the increased need for oxygen during the exercise. We do not think that the lower figure for the oxygen content of the venous blood after exercise can be attributed to the fact that no freezing was used in this instance (Table I).

Experiment 8. Normal Resting Individual after Inspiration of Air with a Low Oxygen Content.—The subject, the same as in Experiment 1, was made to rest quietly on a bed for 10 minutes, after which time he started to breathe from a spirometer which contained a gas mixture of the following composition: oxygen, 5.88 per cent; carbon dioxide 0.13 per cent; and nitrogen 93.99 per cent. The temperature was 16°C.; barometric pressure 776 mm.; oxygen tension 42.77 mm.; and carbon dioxide tension 0.935 mm. 75 seconds after the patient had begun to breathe this mixture the first signs of cyanosis of the lips, ears, cheeks, and fingers

were observed; and after 90 seconds the patient's skin showed general and very marked cyanosis. At this time a sample of venous blood from the right vena mediana cubiti was drawn, and three incisions were made, after previous freezing, in the fourth finger of the left hand. The drawing of the venous blood lasted 10 seconds, whereas it took 2 minutes to collect 5 cc. of cutaneous blood. During the final period of breathing from the spirometer the subject had a very unpleasant feeling of palpitation, air-hunger, and tingling in the extremities, and was very near unconsciousness. After the sample of cutaneous blood was drawn, the subject again started breathing atmospheric air. The respirations were very frequent and very deep, and he felt an immediate relief. The cyanotic skin color disappeared very quickly and was not observed 20 to 25 seconds after beginning to breathe normal air. At that time another sample of cutaneous blood was taken from the incision previously made. It took 30 seconds to collect this blood. The first sample of cutaneous blood showed an unsaturation of 6.9 volumes per cent, which corresponds to 63 per cent saturation. This value is, of course, below the limit for the oxygen content of the arterial blood. On account of the low oxygen tension in the lungs the upper limit for the arterial saturation is much below the total oxygen-combining power. By means of the dissociation curve of the oxyhemoglobin, and the figures for the oxygen tension in the spirometer, we calculate that about 70 per cent would approximate the upper limit for saturation of the arterial blood. Analysis of the venous blood drawn during respiration from the spirometer showed almost the same amount of oxygen that was found in the cutaneous blood. This is probably due to the fact that the venous blood was drawn during a very short period in the first part of the experiment, whereas the cutaneous blood also contains blood from the last part of the experiment; that is, during the time that the arterial unsaturation had reached its highest value. On the other hand, it is not unlikely that the deoxidation in the capillaries was relatively small on account of increased blood flow. Half of the sum of the oxygen unsaturation of the cutaneous (arterial) and the venous blood is 8 to 9 volumes per cent, which is well above the threshold value at which cyanosis usually appears (10). That the cyanosis was found to be generalized is in accordance with its arterial (pulmonary) pathogenesis. The fact that cyanosis of pulmonary origin is more intense in some regions than in others is due to local conditions; for instance, a greater degree of deoxidation in some regions than in others, a richer network of superficial capillaries, a larger filling of the capillaries with blood, or more opened up capillaries (A. Krogh).

In the second cutaneous blood sample normal saturation was found. The change from a marked cutaneous unsaturation to normal condition took place in less than $\frac{1}{2}$ minute after the subject started to breathe normal air. This shows that the circulation of the blood must have been very rapid and that the alveoli of the lungs immediately were supplied with oxygen-rich air. The experiment is in accordance with a similar one done by one of us in 1919 on the same subject (10). No cutaneous blood was drawn at that time.

DISCUSSION.

From the experiments it is evident that the oxygen content of the cutaneous blood and of the arterial blood may show almost identical values. Not only was this true of normal individuals before and after exercise, but it was also found to hold under different pathological conditions. This is, as far as we can see, sufficient to show that, at any rate in some cases, we might obtain similar information from samples of cutaneous and of arterial blood. In the description of the experiments several examples were given of the kind of problem in which examination of cutaneous blood would be of value. We think that in cases in which it is necessary to repeat the blood examination very often, or when for some reason or other it is impossible to obtain arterial blood, it will be especially useful to know that the same information may be obtained by analyzing cutaneous blood. On the other hand, it is admitted that it is not an easy thing to obtain 2 to 3 cc. of blood from an incision in the finger-tip without using stasis. Rather large, and, if freezing is not applied, somewhat painful incisions will be necessary. Two improvements would, we think, be necessary to make the method of general use: one, to perform the analyses for oxygen content with smaller amounts of blood, for instance 0.2 cc. instead of 1 cc. for each analysis as in Van Slyke's apparatus (in this case less than 0.5 cc. would be necessary for other duplicate determinations; such small amounts are, of course, sufficient if the differential method is used which we have employed in only one instance—No. 1); the other improvement would consist in finding a substitute for the method of drawing the blood under oil, for this procedure makes it necessary to draw a larger amount of blood than is needed for the analyses. As to the first point, it will be necessary to find out how much we can decrease the amount of blood taken from the incision and still obtain the same results. As to the second point, we have so far been unsuccessful in developing a method of drawing a small amount of blood from a cutaneous incision directly into a small tube without having the blood come into contact with the air.

Attention should be called to a problem which is not only of theoretical but also of practical importance. It is whether the blood which can be obtained from the cutaneous incision, and which we in

our cases found to be identical with the arterial blood, is genuine arterial blood, that is practically all coming from the arteries themselves, or whether it is to be looked upon as only functional arterial blood, that is arterial blood mixed with a considerable amount of non-deoxidized blood from the capillaries and veins. Both explanations might be correct. An incision in the tip of the finger necessarily severs a number of vessels including small arteries, capillaries, and small veins. It is, however, *a priori* not impossible that after the incision is made, the same conditions would be present that we would find if a slanting water pipe was severed through which water flows from a reservoir with high pressure to a reservoir with low pressure. After cutting such a pipe the water would, of course, only flow out from that part of the pipe that is connected with the reservoir with high pressure. The water in the other part of the pipe would continuously flow away from the place where the pipe was cut. It is known that the statical and other conditions in the circulatory system are different from the conditions in a water pipe, but still it is not an absurdity to suppose that the negative, or at any rate always very low pressure in the central thoracic veins can prevent anything but a negligible amount of blood coming out from the venous side of the severed vessels. The tendency of the veins to collapse would probably act in the same direction. In any case, if no stasis is applied, the difference in pressure in the direction of the blood flow in the arterial and venous system must necessarily bring it about that by far the largest amount of blood taken from the cutaneous incision comes from the arteries. On the other hand, immediately after the incision is made, some blood must necessarily come from the severed veins and capillaries. This blood is probably very quickly washed out from the incision and is contained in the first drop of blood coming out. We have in some, but not in all instances noticed that the first drop of blood coming from the incision is dark, and the following drops very light, indicating that the first drop contains a not negligible amount of venous blood. Even if one makes it a rule, as we have done, to discard in each experiment the first drop of blood, it is possible that a very small amount of blood drawn might contain enough venous blood to make an appreciable difference between the cutaneous and arterial blood. The smallest amount we have taken

has, as mentioned, been 2 cc. The second explanation was that blood obtained from a contaneous incision is derived not only from the arteries, but also, to a considerable extent, from capillaries and veins. It is therefore mixed blood. There are two explanations of the fact that it is arterial in respect to its oxygen content. In the first place, this can be due to a vasodilatation of the region where the incision is made. It is a well recognized fact (1) that mechanical, chemical, or thermal irritants applied to the skin result in a dilatation of the vessels. An increase in the local blood flow and a decrease in local deoxidation must necessarily follow such a vascular dilatation. If an incision is made in such a hyperemic region, quite apart from whether the blood comes from the arteries, capillaries, or veins, it is quite likely that the blood will not differ appreciably from the arterial blood. In the experiments, the finger-tips were in all instances cleaned with alcohol, from 5 to 15 minutes before the incision was made. Furthermore, in some instances (Table I) the skin was frozen before incision was made. The figures in Table I seem to indicate that previous freezing did not have any influence on the results. Whether or not the rubbing with alcohol could be made responsible for the high oxygen content of the cutaneous blood, we are unable to say; but it does not seem very likely, because the alcohol, in some instances, was applied several minutes before the incision. It seems to be more likely that if a vasodilatation takes place, it might be due to the incision itself. An arterialized state of the cutaneous blood could, however, be caused in quite another way. The ratio between the capillary blood flow and the metabolism of the skin might *normally* be very high, so high that no appreciable deoxidation per blood unit takes place. If this were the case, we could explain not only the fact that the oxygen content of the cutaneous blood equals that of the arterial blood, but also an (unpublished) observation we have made in several instances that cyanosis often seems to follow the arterial unsaturation more closely than it does the venous. Such a condition would not be difficult to bring into harmony with one of the chief functions of the skin—the regulation of the temperature. The main difference between the arterial and venous blood of the skin should then be the temperature and not the content of substances relating to the metabolism. On the other hand,

the fact that the first drop of cutaneous blood is often darker than the rest argues against this explanation. The peripherally caused form of cyanosis, that is the cyanosis due to increased deoxidation of capillary blood, also points against such an assumption of the skin circulation.

At first glance it would also seem quite contradictory to this last explanation that the venous arm blood was normally deoxidized in our experiments. No definite conclusions can be drawn from this, however, because the cubital vein drains from other regions than this from which the blood is drawn. Whether one or the other of these explanations contains the truth, it is, at this time, impossible to say.

The identity between the arterial and cutaneous blood has, in the cases examined, only been proved to be true for the oxygen content. There can, however, be little doubt that identity can be extended to include other substances in the blood which pass the capillary wall, for instance salt, sugar, uric acid, etc., because we think that oxygen is the finest indicator of the metabolic interchange between the tissues and the capillary blood. Probably this identity also includes the reaction of the blood which, as is well known, differs in the arteries and veins.

SUMMARY.

1. A procedure is devised whereby cutaneous blood (so called capillary blood) from a finger-tip can be obtained for gas analyses without coming in contact with the air.

2. Determination was made of the oxygen content of the arterial, cutaneous, and venous blood respectively from a normal resting individual, the arterial and cutaneous blood showing the same oxygen content (97.5 and 96.6 per cent of the total oxygen-combining power of the blood). Venous blood drawn simultaneously was 75 per cent saturated.

3. Using the fact that there is a maximum value for the oxygen-combining power of the blood, we have shown, without doing arterial puncture, that under different conditions (normal and pathological individuals, resting and after exercise) the cutaneous blood and the arterial blood are almost identical as far as the oxygen content is concerned.

4. We think that we are justified in extending the identity, found between the oxygen content of the arterial and cutaneous blood, to

other substances in the blood, for instance sugar, salt, uric acid, etc., and also to the reaction of the blood.

5. We are unable to say whether this identity between cutaneous and arterial blood is always true; for example, in a patient with increased venous pressure.

6. In all the experiments we have discarded the first drop of blood, which in some instances was darker than the rest, and always used at least 2 cc. of cutaneous blood. Whether the same results would be obtained with a very small amount of blood, for instance 0.2 to 0.4 cc., we do not know.

7. The experiments show that unless the perfusion of the skin has been extremely great during the experiment, samples of blood obtained from an incision in the skin (of the finger) cannot represent the true capillary blood. The neutral expression cutaneous blood seems therefore for the present preferable to the term capillary blood for samples of blood obtained by cutaneous incisions.

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STUDIES ON THE BIOLOGY OF STREPTOCOCCUS.

II. ANTIGENIC RELATIONSHIPS BETWEEN STRAINS OF STREPTOCOCCUS HÆMOLYTICUS ISOLATED FROM SCARLET FEVER.

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In a previous publication, the author (1) has set forth the results of a study of the agglutination reactions of twenty-five strains of *Streptococcus hæmolyticus* isolated from the throats of patients suffering from scarlet fever. Of these strains, twenty were agglutinated at equal titers by four separate immune sera prepared by the immunization of rabbits to individual strains of scarlatinal streptococci. With the exception of four strains, none was agglutinated by any of four antistreptococcic sera obtained by immunization of animals to strains of *Streptococcus hæmolyticus* isolated from diseases other than scarlet fever. These facts suggest that certain hemolytic streptococci found in the throats of scarlet fever patients constitute a single biological group.

The simultaneous studies of Tunncliffe (2) reveal a similar relationship between strains of *Streptococcus hæmolyticus* associated with scarlet fever. More recently, Gordon (3) has found that eighteen strains of hemolytic streptococcus isolated from scarlatina are identical in their agglutination reactions. None of these strains absorbed the agglutinins from immune sera prepared from certain other types of hemolytic streptococcus, designated by him as Types I and II. On the basis of this evidence, Gordon concludes that the streptococci from the throat secretions in scarlet fever constitute a group serologically distinct from other varieties of *Streptococcus pyogenes*.

The occurrence of hemolytic streptococcus in the throats of patients with scarlet fever is well known and it seems unnecessary to review the literature on the subject at the present time. The most recent observations indicate that *Streptococcus hæmolyticus* is almost constantly

associated with this disease. In the author's series of cases hemolytic streptococcus has been obtained from the throats of 100 per cent of all patients cultured during the 1st week of illness. In those individuals from whom cultures were taken during the first few days of the disease the above organism was found to predominate on the plates. After the 1st week of the disease the number of hemolytic streptococci diminishes rapidly, although great numbers may still be present as late as the 12th day. The average length of time that these organisms are carried in the throat secretions is 10 days. Several examples of the carrier condition have been encountered, and in one individual the specific streptococcus was found to be present in considerable numbers in the throat 1 year after the attack of scarlet fever.

No morphological or cultural characteristics that seem to differentiate the hemolytic streptococcus associated with scarlatina from other types of *Streptococcus pyogenes* have been demonstrated. Many observers have emphasized the variability of the scarlet fever streptococci, and because of this have regarded them as secondary invaders. Such variations as occur seem in all likelihood to be related to inconsistency of the cultural environment rather than to fundamental biological differences. The more recent studies of this organism indicate that differences of this character are of very little value in dividing the group into its specific varieties.

The organisms which have been examined in the present series have been characterized on isolation by wide variations in growth characters.

Some have been very minute and others relatively large. Some grow in short chains, others in long ones. A curious tendency to loss of Gram positiveness has been observed. Although the organisms originally obtained from the peritoneal exudates of mice or from blood agar plates retain the Gram stain, the same strains in transplants from old stock cultures or after cultivation in broth may be continuously Gram-negative. Certain strains manifest a tendency to develop so called involution forms on cultivation in broth media. Many of the individual cocci become from two to three times their original size and appear swollen. Sometimes a culture will become almost entirely converted into forms of this character. Cultivation of such strains in enriched media or even animal passage fails to restore the normal morphology. Coincident with this change in size the strain may become inagglutinable in an immune serum which previously agglutinated it in high titer. One strain (SH26) exhibited this irregular morphology

from the time of isolation and was continuously inagglutinable. The appearance of the colony on blood agar plates is fairly constant, a small moist slightly raised colony with a smooth margin, appearing through a hand lens as an evenly, finely granular dewdrop. About the colony there is a zone of complete hemolysis, of about the same width as the diameter of the colony. The extent of hemolysis, however, varies with different conditions of the media. In some instances the colonies are extremely minute with wide coalescing areas of hemolysis, and in others the zone of hemolysis is quite narrow—from one-half to one-third of the diameter of the colony. Subsequent cultivations indicate that these differences are not constant and that they are dependent upon slight unexplained variations in media or in growth activity.

The hemolytic streptococci from scarlet fever show the same tendency to granular growth in broth that is such a constant feature of other types of *Streptococcus hemolyticus*. Great variations, however, in this characteristic are observed.

All strains when first isolated tend to flocculate when cultivated in broth or when exposed for a few hours in the water bath to a temperature of 55°C. Most strains will grow diffusely in broth after repeated transfers in this medium. Some require relatively few transfers and others from twenty to thirty passages before stable suspensions can be obtained. Certain strains remain persistently granular even after daily passage in broth media for long periods of time. Occasionally a strain will grow diffusely for a while and then again become granular although there has been no recognizable change in the character of the culture medium or in the method of handling the organism. Once an organism acquires the character of diffuse growth sufficiently stable suspensions can usually be prepared to carry out the agglutination reaction at 55°C.

Certain qualities of the medium promote diffuseness of growth.

The addition of glucose or of ascitic fluid causes the cultures to grow luxuriantly and diffusely at incubator temperature, but when such organisms are exposed to normal serum, in the water bath at 55°C., they tend to flocculate, obscuring the results of the agglutination tests. It has been found that flocculation occurs when the sugar concentration is above 0.2 per cent, and that when the concentration is below 0.1 per cent the organisms fail to grow sufficiently to give a satisfactory suspension. Slight variations in the sodium chloride or protein content of broth have no demonstrable effect on the luxuriance or diffuseness of growth. In broth made of meat extract the organisms grow poorly or flocculate, and any appreciable variation from a final hydrogen ion concentration of the medium after sterilization of pH 7.5 results in insufficient growth or flocculation, or usually both. Certain difficulties have been traced to the use of tap water instead of distilled water in the preparation of the medium. It has been found that the addition of 1 or 2 drops of boiled tomato juice to a tube of broth (as used by Thjötta

and Avery (4)) facilitates the growth of the organisms, and does not interfere with agglutination. Passage of autoagglutinable strains through mice sometimes renders them diffuse, but because of the observations of Aronson (5), Meyer (6), and others, that passage through animals makes cultures less agglutinable, the strains used in the present experiments were not passed through animals but grown continuously in broth.

A great variety of fermentation reactions in carbohydrate broth of hemolytic streptococci isolated from the throats of scarlet fever patients has been recorded by different workers. This has led many to believe that they were dealing not with a single type but with a great number of varieties of *Streptococcus hæmolyticus*.

It is, of course, a question how much significance should be attached to these differences in reaction to sugars, particularly in view of the lack of uniformity of the methods employed. Using for convenience the nomenclature of Holman (7) in his classification of streptococci on a basis of carbohydrate fermentation reactions, and considering only strains of hemolytic streptococci isolated from the throats of scarlet fever patients, we find, for example, that Ruediger (8) in a study of thirty-seven such strains, groups some as *Streptococcus infrequens* (lactose, salicin, mannitol fermenters), and others as *Streptococcus pyogenes* (lactose, salicin, non-mannitol fermenters); Kligler (9) encountered *Streptococcus infrequens* and Andrewes and Horder (10) found ten strains of *Streptococcus anginosus* (lactose, non-salicin, non-mannitol fermenters) and three of *Streptococcus subacidus* (non-lactose, non-salicin, non-mannitol fermenters). Floyd and Wolbach (11) report eleven strains of *Streptococcus anginosus* and five of *Streptococcus subacidus*. Similar results have been reported by other investigators. Tunnicliff's (2) strains were with few exceptions *Streptococcus pyogenes*. These rough classifications do not take into account many differences in reaction to saccharose, raffinose, and other less commonly used sugars.

EXPERIMENTAL.

Carbohydrate Fermentation.

The carbohydrate fermentation reactions of the present series of throat strains are presented in Table I.

It is seen that of 64 throat strains examined, 53 (82.8 per cent) are true *Streptococcus pyogenes* (Holman), and if one accepts the opinion of Gordon and others that certain strains of *pyogenes* occasionally ferment mannitol, one may be justified in assuming that six additional strains, or 59 in all (92 per cent), are *Streptococcus pyogenes*. Of the

TABLE I.

Carbohydrate Fermentation by Strains of Hemolytic Streptococci Isolated from the Throats of Scarlet Fever Patients.

Strain No.	Lac-tose.	Salicin.	Man-nitol.	Inulin.	Raffi-nose.	Strain No.	Lac-tose.	Salicin.	Man-nitol.	Inulin.	Raffi-nose.
11	+	+	-	-	-	74	+	+	-	-	-
12	+	+	-	-	-	75	+	+	-	-	-
13	+	+	-	-	-	76	+(?)	+	-	-	-
14	+	+	-	-	-	77	+	+	-	-	-
15	+	+	+	-	-	78	+	+	-	-	-
16	+	+	-	-	-	79	+	+	-	-	-
20	+	+	-	-	-	80	+	+	-	-	-
23	+	+	-	-	-	81	+	+	-	-	-
24	+	+	-	-	-	82	+	+	-	-	-
25	+	+	+	-	-	83	+	+	-	-	-
26	+	+	-	-	-	84	+	+	-	-	-
27	+	+	-	-	-	88	+	+	-	-	-
28	+	+	+	-	-	89	+	+	-	-	+
29	+	+	+	-	-	90	+	+	-	-	-
30	+	+	-	-	-	91	+	+	-	-	-
35	+	+	+	-	-	93	+	+	-	-	-
36	-	+	-	-	-	94	+	+	-	-	-
38	+	+	-	-	-	95	+	+	-	-	-
39	+	+	-	-	-	98	+	+	+	-	-
45	+	+	-	-	+	99	+	+	-	-	-
47	+	+	-	-	-	201	+	+	-	-	-
50	+	+	-	-	-	203	+	+	+	-	+
53	+	+	-	-	-	205	+	+	-	-	-
54	+	+	-	-	-	207	+	+	-	-	-
56	+	+	-	-	-	208	+	+	-	-	-
62	+	+	-	-	+	209	+	+	-	-	-
65	+	+	-	-	-	210	+	+	-	-	-
66	+	+	-	-	-	211	+	+	-	-	-
67	+	+	-	-	-	212	+	+	-	-	-
68	+	+	-	-	-	213	+	+	-	-	-
69	+	+	-	-	-						
70	+	+	-	-	-						
72	+	+	-	-	-						
73	+	+	-	-	-						

0.1 per cent carbohydrate meat infusion broth was used containing Andrade indicator. Incubated 1 week; readings made daily.

+ indicates fermentation, - no fermentation.

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remaining five strains, four ferment raffinose, which puts them in the group of *Streptococcus salivarius*, presumably a saprophytic type. It is interesting to note in this connection that, as shown below, three of these raffinose-fermenting strains differ in their reactions to agglutinating sera from the other strains. In brief, it may be concluded from these results that most of the strains belong to the type of *Streptococcus pyogenes*, and do not differ in their carbohydrate fermentation reactions from strains isolated from acute tonsillitis, pharyngitis, erysipelas, and certain septic processes. On the other hand, our experience does not demonstrate the existence of a great variety of carbohydrate-fermenting types. Some strains are slow fermenters, and it may be that some of the variations previously reported are due to incomplete observations. Studies with the less common sugars have not been made.

Agglutination Tests.

Numerous investigators have studied the agglutination reactions of streptococci. The results on the whole have been rather conflicting due largely no doubt to differences of method and inherent technical difficulties.

Moser (12) and Moser and von Pirquet (13) have prepared polyvalent sera from horses, using the streptococcus of scarlet fever as antigen. They studied the capacity of such sera to agglutinate specifically various strains of scarlatinal streptococci. The latter strains were agglutinated in dilutions of 1:1,000 or over, whereas hemolytic streptococci from other sources were not specifically agglutinated. As a consequence of these observations the authors believe that the streptococcus of scarlet fever differs from similar strains isolated from instances of erysipelas, phlegmon, puerperal sepsis, etc. Meyer (6) and later Rossiwall and Schick (14) have in general confirmed the results of Moser and von Pirquet.

On the other hand, Hasenknopf and Salge (15), Aronson (5), and also Neufeld (16) have been unable to duplicate the above results. They failed to find definitely specific relationships between the streptococci of scarlet fever and are of the opinion that it is not possible to differentiate between types of hemolytic streptococci by means of the agglutination reaction.

The discordant results briefly reported are no doubt due in part to technical difficulties encountered in the effort to study specific agglutinations of streptococcus and in part to the variety of methods

employed. The tendency of these organisms to agglutinate spontaneously in normal sera, and in broth and saline suspensions is very confusing. In addition, animal passage and long continued growth in artificial media may induce variations in agglutinability. Moreover, the methods of different investigators have not been strictly comparable. For these reasons it has been impossible up to the present time to answer conclusively the question as to whether or not the streptococci of scarlet fever form a specific immunologic type.

In a recent publication, Dochez, Avery, and Lancefield (17) have described a technique for the agglutination of streptococcus which has made it possible to differentiate between types of hemolytic streptococci isolated from a variety of respiratory diseases. Their procedure has been used in the present study. Its essential points are the use for purposes of culture of a meat infusion broth the reaction of which remains at or near pH 7.5. No sodium chloride is added and in such medium most strains of streptococcus can be grown diffusely. Broth of the same character and reaction is used in all the necessary dilutions both of organisms and of sera. The test suspensions are incubated for $1\frac{1}{2}$ hours in a water bath at 55°C. and then read.

The sera were prepared by inoculating rabbits intravenously at 2 or 3 day intervals with increasing doses of heat-killed broth cultures, starting with 0.2 cc. and increasing to 2 cc. All rabbit sera were tested before the first inoculation in order to guard against spontaneous agglutination by normal rabbit sera. The above method produced sera with a titer of over 1:2,560. It was noted that the serum immediately after separation from the clot showed an agglutinating titer markedly lower than after standing for 48 hours or longer in the ice chest, so only sera have been used which have remained at least 48 hours on ice. Such sera, however, lose their agglutinins after standing for a few months.

The results of the first series of agglutination tests have been reported (1). They are recapitulated in Table II, with the addition of a few strains studied shortly thereafter.

Table II shows that none of five non-scarlatinal antistreptococcic sera (R.Sm.3, R.Sm.23, R.Sm.32, R.Sm.60, and R.Sm.84, the type sera of Dochez, Avery, and Lancefield) agglutinates any of the thirty-three strains of streptococcus isolated by the author from the throats

of scarlet fever patients, with the exception of eight strains agglutinated by Serum R.Sm.84, and Strain 63, agglutinated by Serum R.Sm.32. Each of the scarlatinal streptococcic sera used agglutinates twenty-eight out of thirty-four strains isolated from patients with scarlet fever. It is of some interest that of the six scarlet fever strains not agglutinated by scarlatinal antisera, Strains 36, 45, and 62 differ from the type in their fermentation reactions. Strain 26 persistently showed large numbers of involution forms, a phenomenon characteristic of inagglutinable strains. Strain 54, erroneously included in the series first reported, has been omitted from Table II. In a few instances individual agglutination tests have not been made because of deterioration of the sera during the summer months.

In order to confirm the results reported in Table II an additional series of strains of streptococcus has been isolated from fresh cases of scarlet fever. New sera have been prepared by the immunization of rabbits and the specific agglutinating power of these sera has been tested. The results are summarized in Table III.

Of the twenty-four new strains studied, twenty are agglutinated specifically by antiscarlatinal sera. One strain (No. 79) displays some irregularity of agglutination, and three (Nos. 84, 203, and 205) fail completely to agglutinate. If the statistics reported in Tables II and III are combined it is found that 80 per cent or more of strains of streptococcus from scarlet fever are agglutinated by the specific sera employed. In all, the various strains were tested with ten different monovalent antistreptococcic sera. On the other hand, these same strains are not agglutinated by antistreptococcic sera prepared from hemolytic streptococci isolated from diseases or conditions other than scarlet fever.

A study of the capacity of immune sera prepared from the scarlatinal streptococcus to agglutinate streptococci from a variety of sources has also been made. The results of this study are shown in Table IV.

This table demonstrates that streptococci isolated from individuals not having scarlet fever or removed from contact with this disease do not agglutinate in antiscarlatinal sera. Of the five strains showing agglutination (Nos. 112, D10, 10, 55, and 57), one (No. 112) was from a chronic carrier, two (Nos. 10 and D10) were from recent contacts,

one (No. 55) from an infected mastoid shortly before the development by the patient of a typical scarlatinal rash followed by desquamation, and one (No. 57) from the throat of an individual suspected of having scarlet fever. In general then it would seem that hemolytic streptococci isolated from scarlet fever constitute a specific type which can be separated by the reaction of agglutination from hemolytic streptococci from other sources.

A number of studies have previously been made of the power of the serum of convalescents from scarlet fever to agglutinate the homologous streptococcus. The results have been irregular and conflicting and are of no great significance. In spite of these apparent failures to demonstrate a positive agglutination reaction the author has studied a few such sera. The results are presented in Table V. The degree of agglutination obtained in a few instances is considerable but the range so limited that positive deductions as to specificity are unwarranted.

Absorption of Agglutinin Tests.

Specific absorption of agglutinins by streptococcus is a procedure attended by certain technical difficulties. The high temperature at which the reaction must be carried out causes a considerable loss of agglutinating power by the immune sera employed. The use, however, of a very heavy suspension of bacteria for purposes of absorption so shortens the period of incubation that no great deterioration occurs. The sediment of 50 cc. of a 24 hour broth culture of streptococcus was added to each cubic centimeter of a dilution of 1:5 of the serum to be absorbed. The mixtures were incubated at 55°C. for 2 hours with frequent shaking and then the organisms were removed by centrifugalization. The normal sera used as controls were treated for a similar period of time. The results are shown in Table VI.

From this table it is seen that a scarlet fever streptococcus removes from an homologous serum the agglutinins for the particular strain used in its production and for other scarlet fever strains as well. On the other hand, a non-scarlatinal streptococcus does not absorb these agglutinins. Furthermore, a scarlatinal streptococcus fails to absorb the agglutinins from a serum prepared by immunization to a

TABLE III.

Agglutination of Hemolytic Streptococci from the Throats of Scarlet Fever Patients with Monodent Scarlatinal and Non-Scarlatinal Antistreptococcic Sera. Series 2.

Scarlatinal streptococcus strains.	Antistreptococcic sera.									
	Scarlatinal sera.						Non-scarlatinal sera.			
	Sm.15a.	Sm.15b.	Sm.25.	Sm.53a.	Sm.53b.	Sm.76a.	Sm.76b.	Sm.102.	R.Sm.60.	
74	++	++	++	++	++	++	+	—	0	
75	++	++	++	++	++	++	++	—	0	
76	++	++	++	0	++	++	++	—	0	
77	++	++	++	++	++	++	0	—	0	
78	++	++	++	—	++	++	++	—	0	
79	—	0	++	0	0	—	0	—	0	
80	++	++	++	++	++	++	++	—	0	
81	++	++	++	++	++	++	++	—	0	
82	++	++	++	++	++	++	++	—	0	
83	—	++	++	++	0	++	++	—	0	
84	—	—	—	—	—	—	0	—	0	
88	++	++	++	++	++	++	++	—	0	
89	++	++	++	++	++	++	++	—	0	
90	++	++	++	++	++	++	0	—	0	
91	++	++	++	++	++	0	0	—	0	
93	++	++	0	++	0	++	0	—	0	

TABLE IV.

Agglutination of Hemolytic Streptococci from Non-Scarlatinal Sources by Monovalent Scarlatinal Antistreptococic Sera.

Non- scarlatinal strepto- coccus strains.	Source.	Scarlatinal antistreptococic sera.			
		Sm.15a.	Sm.25.	Sm.53a.	Sm.76.
63	Blood culture, erysipelas.	-	-	-	0
101	Pus, osteomyelitis.	+++	-	-	0
103	" maxillary sinusitis.	-	-	-	0
104	" abscess of finger.	-	-	-	0
105	Cervical culture, puerperal sepsis.	0	-	-	0
106	Pus, abscess.	-	-	0	-
115	" empyema.	-	-	-	0
102	Tonsillitis.	-	-	0	-
111	"	-	-	+	0
112	"	+++	++	++	0
113	"	+	-	-	0
114	"	-	-	-	0
D1	Normal throat, scarlet fever contact.	-	-	-	-
D3	" " " "	-	-	-	-
D4	" " " "	-	-	-	-
D5	" " " "	-	-	-	-
D6	" " " "	-	-	-	-
D7	" " " "	-	-	-	-
D8	" " " "	-	-	-	-
D9	" " " "	-	-	-	-
D10	" " " "	+++	++	+	+++

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Non-scarlatinal strains previously recorded (1).	Source.	R.Sm.273.	Sm.25.	
4	Pus, cervical abscess.	—	—	
5	" "	—	—	
32	" mastoid.	—	—	
37	Blood culture, septicemia.	—	—	
40	" "	—	—	
49	" " erysipelas.	—	—	
55	Pus, mastoid, prescarlet fever.	+++++	+++++	
58	" "	—	—	
59	" abscess of jaw.	—	—	
6	Tonsillitis.	—	—	
9	" "	—	—	
10	" " (scarlet fever contact).	+++++	+++++	
21	" "	—	—	
22	" "	—	—	
31	" "	—	—	
41	" "	—	—	
57	" " (scarlet fever suspect).	+++++	+++++	

TABLE V.

Agglutination of Hemolytic Streptococcus from the Throats of Scarlet Fever Patients with Scarlatinal Convalescent Sera.

Scarlatinal streptococcus strains.	Convalescent scarlatinal sera.	Dilution.					
		1:8	1:16	1:32	1:64	1:128	1:256
12	Sm.62 (6th day).	—	—	—	—	—	—
24	" 62 (6th ").	—	—	—	—	—	—
25	" 62 (6th ").	—	—	—	—	—	—
28	" 62 (6th ").	—	—	—	—	—	—
11	Sm.62 (18th day).	++	+	—	—	—	—
12	" 62 (18th ").	++	+	+	—	—	—
14	" 62 (18th ").	+	+	—	—	—	—
15	" 62 (18th ").	++	++	+	+	—	—
23	" 62 (18th ").	++	+	+	—	—	—
24	" 62 (18th ").	+	+	—	—	—	—
25	" 62 (18th ").	++	++	+	+	—	—
62	" 62 (18th ")	++	++	++	++	+	—
11	Sm.73 (4th day).	—	—	—	—	—	—
12	" 73 (4th ").	—	—	—	—	—	—
14	" 73 (4th ").	—	—	—	—	—	—
15	" 73 (4th ").	—	—	—	—	—	—
24	" 73 (4th ").	—	—	—	—	—	—
25	" 73 (4th ").	—	—	—	—	—	—
R273	" 73 (4th ").	—	—	—	—	—	—
11	Sm.73 (18th day).	+	+	—	—	—	—
12	" 73 (18th ").	—	—	—	—	—	—
14	" 73 (18th ").	+	+	—	—	—	—
15	" 73 (18th ").	++	++	+	+	—	—
24	" 73 (18th ").	+	+	+	+	—	—
25	" 73 (18th ").	++	++	+	+	—	—
R273	" 73 (18th ").	++	+	+	—	—	—
80	Sm.203 (14th day).	—	—	—	—	—	—
81	" 203 (14th ").	—	—	—	—	—	—
82	" 203 (14th ").	—	—	—	—	—	—
94	" 203 (14th ").	++	+++	+++	+++	++	—
96	" 203 (14th ").	—	—	—	—	—	—
112	" 203 (14th ").	—	—	—	—	—	—
204	" 203 (14th ").	—	—	—	—	—	—
206	" 203 (14th ").	—	—	—	—	—	—
207	" 203 (14th ").	—	—	—	—	—	—
201	Sm.207 (15th day).	—	—	—	—	—	—
202	" 207 (15th ").	—	—	—	—	—	—
204	" 207 (15th ").	—	—	—	—	—	—
206	" 207 (15th ").	—	—	—	—	—	—

In Tables V to VIII, XI, XII, and XIV — indicates no agglutination, ± doubtful agglutination, + slight agglutination, ++ frankly positive agglutination, +++ heavy agglutination, ++++ complete agglutination.

streptococcus from a source other than scarlet fever. Similar results are illustrated by Tables VII and VIII.

The experiments shown in Tables VI to VIII show that it is possible to obtain a specific absorption of agglutinins from antiscarlatinal sera by use of streptococci from scarlet fever. Success, however, did not attend all such efforts and from time to time insurmountable difficulties are encountered.

Protection Tests.

An effort has been made to confirm the specificity of the agglutination reaction of the streptococci from scarlet fever by means of the protection test. Antistreptococcic sera as a rule have a rather low protective value for experimental animals in comparison, for example, with antipneumococcic sera. Previous investigators who have studied this question have reported in some instances a certain degree of specific protection and in others that the protective power of a monovalent serum is manifest against more than one type of streptococcus. In carrying out this test it is important that the serum should have a fairly high protective titer and that the organisms used should be of maximum virulence in order that any single test may extend over a sufficiently wide range of dosage. In the experiments shown in Tables IX and X the methods and conditions prescribed by Dochez, Avery, and Lancefield have been observed.

These experiments show that it is possible to develop in immune sera a certain degree of protective value for white mice against highly virulent strains of streptococcus isolated from scarlet fever. Such protective power is manifest not only against the homologous strain but also against strains from other cases of scarlet fever. Immune sera prepared by the use of strains of hemolytic streptococcus from sources other than scarlet fever possess no such protective power against scarlatinal streptococci.

Study of Contacts with Scarlet Fever.

It has long been recognized that scarlet fever may be propagated by means of carriers and does not require direct contact for its transmission. Sometimes the carrier may be a healthy individual, or may have suffered from a slight sore throat, without developing an exan-

TABLE VI.

Absorption Experiment with Scarlatinal Antistreptococcic Serum, No. 15a, and with Non-Scarlatinal Antistreptococcic Serum, No. 102.

Two specimens of 1 cc. each of Serum Sc.Sm.15a, 1:5, were absorbed with scarlatinal streptococcus, Strain 15 (homologous strain), and with non-scarlatinal streptococcus, Strain 102, respectively, as described above. Specimens of Non-Scarlatinal Serum 102 were similarly absorbed. The experiment follows.

Row.	Streptococcus strains.	Sera.	Dilution.					
			1:40	1:80	1:160	1:320	1:640	1:1,280
1	15	Sc.Sm.15a absorbed with Scarlatinal Strain 15.	—	—	—	—	—	—
2	15	“ “ 15a “ Non-Scarlatinal Strain 102.	+	+	+	+	+	+
3	15	Non-Sc.Sm.102 absorbed with Scarlatinal Strain 15.	—	—	—	—	—	—
4	15	“ “ 102 “ “ Non-Scarlatinal Strain 102.	—	—	—	—	—	—
5	15	Normal rabbit serum.	—	—	—	—	—	—
	15	Plain broth control.	—	—	—	—	—	—
6	201	Sc.Sm.15a absorbed with Strain 15.	—	—	—	—	—	—
7	201	“ “ 15a “ “ 102.	+	+	+	+	+	+
8	201	Non-Sc.Sm.102 absorbed with Strain 15.	—	—	—	—	—	—
9	201	“ “ 102 “ “ 102.	—	—	—	—	—	—
10	201	Normal rabbit serum.	—	—	—	—	—	—
	201	Plain broth control.	—	—	—	—	—	—
11	112	Sc.Sm.15a absorbed with Strain 15.	+	+	+	+	+	+
12	112	“ “ 15a “ “ 102.	+	+	+	+	+	+
13	112	Non-Sc.Sm.102 absorbed with Strain 15.	—	—	—	—	—	—
14	112	“ “ 102 “ “ 102.	—	—	—	—	—	—
15	112	Normal rabbit serum.	—	—	—	—	—	—
	112	Plain broth control.	—	—	—	—	—	—

16	102	Sc.Sm.15a absorbed with Strain 15.	-	±	-	-	-	-	-
17	102	" " 15a " " 102.	±	+	-	-	-	-	-
18	102	Non-Sc.Sm.102 absorbed with Strain 15.	+	+	+	+	+	+	+
19	102	" " 102 " " 102.	+	+	-	-	-	-	-
20	102	Normal rabbit serum.	+	+	-	-	-	-	-
	102	Plain broth control.	-	-	-	-	-	-	-
21	114	Sc.Sm.15a absorbed with Strain 15.	-	-	-	-	-	-	-
22	114	" " 15a " " 102.	-	-	-	-	-	-	-
23	114	Non-Sc.Sm.102 absorbed with Strain 15.	-	-	-	-	-	-	-
24	114	" " 102 " " 102.	-	-	-	-	-	-	-
25	114	Normal rabbit serum.	-	-	-	-	-	-	-
	114	Plain broth control.	-	-	-	-	-	-	-

Strains 15 and 201 were isolated from scarlatinal throats, No. 112 from the probable carrier referred to above, and Nos. 102 and 114 from throats in cases of tonsillitis.

TABLE VII.

Absorption Experiment with Scarlatinal Antistreptococcic Serum, No. 76a.

Two specimens of 1 cc. each of scarlatinal antistreptococcic serum, No. 76a, were absorbed with scarlatinal streptococcus, Strain 15, and with non-scarlatinal streptococcus, Strain 102, respectively.

Row.	Streptococcus strains.	Sera.	Dilution.					
			1:40	1:80	1:160	1:320	1:640	1:1,280
1	15	Sc.Sm.76a absorbed with Scarlatinal Strain 15.	—	—	—	—	—	—
2	15	“ “ 76a “ “ Non-Scarlatinal Strain 102.	+++	+++	+++	+++	++	—
3	15	“ “ 76a not absorbed.	+++	+++	+++	+++	+++	+
4	15	Normal rabbit serum.	—	—	—	—	—	—
5	15	Plain broth control.	—	—	—	—	—	—
6	201	Sc.Sm.76a absorbed with Scarlatinal Strain 15.	—	—	—	—	—	—
7	201	“ “ 76a “ “ Non-Scarlatinal Strain 102.	+++	+++	+++	+++	++	—
8	201	Normal rabbit serum.	+++	+++	+++	+++	+	—
9	201	Plain broth control.	—	—	—	—	—	—
10	112	Sc.Sm.76a absorbed with Scarlatinal Strain 15.	—	—	—	—	—	—
11	112	“ “ 76a “ “ Non-Scarlatinal Strain 102.	+++	+++	+++	+++	++	—
12	112	Normal rabbit serum.	+++	+++	+++	+++	++	—
13	112	Plain broth control.	—	—	—	—	—	—
14	117	Sc.Sm.76a absorbed with Scarlatinal Strain 15.	—	—	—	—	—	—
15	117	“ “ 76a “ “ Non-Scarlatinal Strain 102.	++	+	+	+	—	—
16	117	Normal rabbit serum.	+++	+++	+++	+++	—	—
17	117	Plain broth control.	—	—	—	—	—	—

17	102	Sc.Sm.76a absorbed with Scarlatinal Strain 15.	-	-	-	-	-	-
18	102	" " 76a " " Non-Scarlatinal Strain 102.	-	-	-	-	-	-
19	102	" " 76a not absorbed.	-	-	-	-	-	-
20	102	Normal rabbit serum.	-	-	-	-	-	-
	102	Plain broth control.						
21	114	Sc.Sm.76a absorbed with Scarlatinal Strain 15.	-	-	-	-	-	-
22	114	" " 76a " " Non-Scarlatinal Strain 102.	-	-	-	-	-	-
23	114	" " 76a not absorbed.	+	-	-	-	-	-
24	114	Normal rabbit serum.	-	-	-	-	-	-
	114	Plain broth control.						

Strains 15 and 201 were isolated from the throats of scarlet fever patients, Nos. 112 and 117 from the throat of the carrier, and Nos. 102 and 114 from throats in cases of tonsillitis.

TABLE VIII.

*Absorption Experiment with Scarlatinal Antistreptococcic Serum, No. 15a, Absorbed with Scarlatinal Carrier Streptococcus, Strain 112, and Non-Scarlatinal Streptococcus, Strain 114.**

Row.	Streptococcus strains.	Sera.	Dilution.					
			1:40	1:80	1:160	1:320	1:640	1:1,280
1	15	Sc.Sm.15a absorbed with scarlet fever carrier strain, No. 112.	—	—	—	—	—	—
2	15	“ “ 15a “ “ Non-Scarlatinal Strain 114.	++	++	++	++	++	—
3	15	“ “ 15a not absorbed.	++	++	++	++	++	++
4	15	Normal rabbit serum.	++	++	++	++	++	++
	15	Broth.	—	—	—	—	—	—
5	112	Sc.Sm.15a absorbed with scarlet fever carrier strain, No. 112.	—	—	—	—	—	—
6	112	“ “ 15a “ “ Non-Scarlatinal Strain 114.	+	++	++	++	++	—
7	112	“ “ 15a not absorbed.	++	++	++	++	++	+
8	112	Normal rabbit serum.	++	++	++	++	++	—
	112	Broth.	—	—	—	—	—	—
9	114	Sc.Sm.15a absorbed with scarlet fever carrier strain, No. 112.	—	—	—	—	—	—
10	114	“ “ 15a “ “ Non-Scarlatinal Strain 114.	—	—	—	—	—	—
11	114	“ “ 15a not absorbed.	—	—	—	—	—	—
12	114	Normal rabbit serum.	—	—	—	—	—	—
	114	Broth.	—	—	—	—	—	—

*The same technique was used as in Table VII.

TABLE IX.

Protection against Virulent Scarlatinal Streptococcus, Strain 25, with Antistreptococcic Sera C13 and 53.*

The mice were inoculated in the afternoon of the 1st day intraperitoneally with 0.5 cc. each of the sera, and on the morning of the 2nd day with the graduated doses of the virulent streptococcus, No. 25, as indicated.

Mouse No.	Serum.	Culture.	Result.
		cc.	
1	None.	0.0001	Died in 30 hrs.
2	"	0.00001	" " 36 "
3	"	0.000001	Very sick for 2 days, but recovered.
4	0.5 cc. of Serum Sm.C13.	0.001	Died in 36 hrs.
5	0.5 " " " " C13.	0.0001	Survived.
6	0.5 " " " " C13.	0.00001	"
7	0.5 " " " " C13.	0.000001	"
8	0.5 " " " " 53.	0.001	"
9	0.5 " " " " 53.	0.0001	"
10	0.5 " " " " 53.	0.00001	"
11	0.5 " " " " 53.	0.000001	"

*Strain 25 had been so raised in virulence through twenty-eight mouse passages that less than 0.00001 cc. of a blood broth culture killed a mouse in less than 48 hours.

TABLE X.

Protection against Virulent Scarlatinal Streptococcus, Strain 25, with Scarlatinal Antistreptococcic Sera, Nos. 15 and 76.*

Mouse No.	Serum.	Culture.	Result.
		cc.	
1	None.	0.0001	Died in 72 hrs.
2	"	0.00001	" " 36 "
3	"	0.000001	" " 48 "
4	0.5 cc. of Serum Sm.15.	0.01	" " 24 "
5	0.5 " " " " 15.	0.001	Survived.
6	0.5 " " " " 15.	0.0001	Died in 70 hrs. (Peritoneal fluid sterile.)
7	0.5 " " " " 15.	0.00001	Died in 76 hrs.
8	0.5 " " " " 76.	0.01	" " 72 "
9	0.5 " " " " 76.	0.001	Survived.
10	0.5 " " " " 76.	0.0001	Died in 28 hrs.
11	0.5 " " " " 76.	0.00001	Survived.

*The strain and the technique used were the same as in Table IX.

them. An unusual opportunity to study such a set of conditions was afforded by a localized outbreak of scarlet fever in a practically isolated community.¹

Beyond the outskirts of the little village of Claiborn, on the east shore of the Chesapeake Bay, the Maryland Tuberculosis Association maintains a fresh air home, called Claiborn Cottage, where non-tuberculous children of tuberculous parents, children below par physically, but with no physical signs of tuberculosis, are sent for several weeks or months during the summer. The cottage is situated directly on the shore of the bay and is surrounded on the remaining sides by dense woods. No parents or friends visit the cottage. At the time of the outbreak, there were in the cottage thirty-three children, varying from 3 to 12 years of age, and two nurses and four colored helpers. On September 21, 1920, Wm. W., age 8, was taken ill with general malaise and a slight sore throat. He was immediately isolated. Later headache, vomiting, red throat, and flushing of the skin developed. On the morning of the 4th day (September 24) he exhibited a typical scarlatini-form erythema over the face, chest, and body. He was then transferred to the Sydenham Hospital. 2 days later (September 26) Edw. M., age 4, was taken ill with vomiting. He, too, was promptly quarantined in a separate room. 2 days later he developed a typical angina and strawberry tongue. Simultaneously, on September 26, Jas. L., age 12, also became ill, and complained of headache and slight sore throat. He was also quarantined and 2 days later showed a typical scarlatinal rash. Edw. M. and Jas. L. were then sent to Sydenham Hospital, and on the day following, Edw. M. exhibited a scarlatiniform rash. All three patients subsequently experienced typical attacks of uncomplicated scarlet fever, followed by desquamation. The author visited the three boys at the Sydenham Hospital and isolated strains of hemolytic streptococcus from the throats of all. No other case of scarlet fever developed at the Claiborn Cottage.

The question then arose as to the source of infection. It could not have been the neighboring village, since the children were never allowed to leave the premises nor did the villagers ever visit the cottage. Even the servants lived in a small cottage on the grounds. Furthermore, there were no cases of scarlet fever reported in that county at the time. The milk supply was beyond suspicion, inasmuch as it came from one small farm nearby, and was handled by only the farmer's immediate family and the staff of the Claiborn Cottage. Careful investigation failed to reveal any history of sore throat, or any indisposition whatsoever among any of the members of the farmer's family or help. The infection was not introduced by visitors since there had been no visitors during the summer. Suspicion was directed, therefore, to the only recent admissions to the cottage, three little

¹ For this opportunity I am indebted to Dr. John F. Hogan of the Baltimore Department of Health, and to Miss Spielman of the Maryland Tuberculosis Association.

brothers, Joseph, Francis, and James B., Russian-Americans, who had been admitted to Claiborn Cottage, September 17, 4 days before the development of the first case of scarlet fever. There had been no other admissions within 3 weeks.

Joseph, Francis, and James B., age 11, 10, and 8 years, at the time of observation were apparently free from any acute infection, and denied having had sore throats recently. However, at the time they left home, September 17, a sister, Julia B., age 12, was ill with a severe sore throat, which had lasted for several days. She visited on September 20 the children's dispensary of the Johns Hopkins Hospital, where it is stated on her record that she had just recovered from an acute tonsillitis. No note was made at that time of the presence of a rash or of desquamation, nor could a history of these be obtained later from the mother.

The cottage was visited by the author on October 1 and cultures were taken on blood agar plates from the throats of the thirty-nine inhabitants. From the throats of twenty-three, including the nurses and helpers, no hemolytic streptococci were recovered. On the other hand, hemolytic streptococci were obtained in greater or less numbers from sixteen of the inmates of the cottage, the largest numbers of colonies being present on the plates from James B., Joseph B., and Francis B., the three recent admissions. None of the latter at any time had sore throat, fever, or rash. An immune serum, No. C13, was prepared by immunization of a rabbit with Strain C13 from Joseph B. The capacity of this serum to agglutinate the group of Claiborn streptococci was then tested. The results of this test are shown in Table XI.

The result of this series of tests demonstrates that none of the strains of hemolytic streptococci isolated from the throats of the children in the Claiborn Cottage was agglutinated by the serum made by immunization of a rabbit to one of these strains (No. C13), except the homologous strain, and also (slightly) Strain C15 (Francis B.). On the other hand, this serum agglutinated seventeen out of twenty strains of scarlatinal streptococci (Table XII). It is of particular interest to note that among these scarlatinal strains were Nos. 76, 77, and 78, the strains isolated from the throats of Wm. W., Edw. M., and Jas. L., the boys who had contracted scarlet fever while at Claiborn Cottage.

Furthermore, the Claiborn strains, with the exception of Nos. C13 and C15, and the irregularly agglutinated Strains C9 and C34, failed to be agglutinated by five scarlatinal antistreptococcic sera. The results of this test are shown in Table XIII.

The series of tests shown in Tables XI to XIII would seem to indicate that hemolytic streptococcus strain, No. C13, carried in the throat of Joseph B. differed from all others found in the throats of the

children at Claiborn, except that from his brother Francis B., Strain C15. Study of its agglutination reactions shows it to be of the same biological type as strains isolated from the great majority of throats in scarlet fever, indicating that it is a true scarlatinal streptococcus.

TABLE XI.

Agglutination of Hemolytic Streptococci from the Throats of Inhabitants of Claiborn Cottage by Serum C13.

Streptococcus strains.	Dilution of Antistreptococcic Serum C13.					
	1:40	1:80	1:160	1:320	1:640	1:1,280
C1	—	—	—	—	—	—
C7	—	—	—	—	—	—
C8	—	—	—	—	—	—
C9	—	—	—	—	—	—
C10	—	—	—	—	—	—
C13	++++	++++	+++	+++	+++	+++
C15	++	+	+	—	—	—
C18	—	—	—	—	—	—
C19	—	—	—	—	—	—
C20	—	—	—	—	—	—
C21	—	—	—	—	—	—
C22	—	—	—	—	—	—
C23	—	—	—	—	—	—
C30	—	—	—	—	—	—
C33	—	—	—	—	—	—
C34	—	—	—	—	—	—

The throats were numbered serially, as cultured, so that the above numbers represent the positive cultures from the thirty-nine throats examined.

Immune sera were also made with Strain 76, the strain isolated from Wm. W., the first case of scarlet fever to develop at Claiborn Cottage. These sera, Sm.76a, and Sm. 76b, agglutinated typically streptococci isolated from other scarlatinal throats. The results have already been tabulated in Table III. They also agglutinated Strain C13 in high dilution, but failed to agglutinate the other Claiborn strains. In short, Serum 76 gave identically the same agglutination reactions as did Serum C13.

A culture was made from the throat of Julia B., the sister of the three boys, and although 2 weeks had elapsed since her attack of tonsillitis, a few colonies of

hemolytic streptococcus were obtained. Strain 79 isolated from this source was agglutinated by one antiscarlatinal serum but not by two other such sera. As the strain quickly became persistently granular, this discrepancy could not be further investigated.

A fourth brother, Louis B., age 9, came to the Johns Hopkins dispensary, November 17, giving a history of having had a cold about October 1, just after

TABLE XII.

Agglutination of Scarlatinal Streptococcus Strains by Serum C13.

Scarlatinal streptococcus strains.	Dilution of Serum C13.					
	1:40	1:80	1:160	1:320	1:640	1:1,280
74	++++	++++	++++	++	+	—
75	++++	++++	++++	++	—	—
76	++++	++++	++++	++++	++++	++++
77	++++	+++	+++	++	++	+
78	++++	++++	++++	++++	++++	++++
79	++	++	++	++	++	++
80	++++	++++	++++	++++	+++	+
81	++++	+++	++	+	—	—
82	++++	++++	++++	++	—	—
83	—	—	—	—	—	—
84	++++	++++	++++	++	+	—
88	+++	+++	+++	++	++	+
89	++++	++++	++++	++++	+++	++
90	+++	++	++	++	+	—
91	+++	++	+	—	—	—
93	++++	++++	++++	++++	+++	+++
94	—	—	—	—	—	—
95	—	—	—	—	—	—
98	+++	+++	++	+	—	—
99	++	++	++	++	+	—

the sister's recovery from sore throat and while the other three brothers were at Claiborn Cottage. His mother noticed that his face was swollen, and that during the following week the swelling extended to his extremities and abdomen. The patient had no angina, rash, or subsequent desquamation of the skin. About October 8, the edema became very marked and he began to have fever and headache. He was kept in bed 2 weeks, during which time the swelling gradually disappeared. The mother then noticed urinary frequency, and on November 17,

the patient was brought to the Johns Hopkins Hospital dispensary. At this time his feet were still a little edematous, and his urine showed a trace of albumin and some casts. His tonsils were enlarged, and cultures from his throat on a blood agar plate yielded a considerable number of colonies of hemolytic streptococcus. The strain isolated is designated C40. The agglutination reactions of this strain are shown in Table XIV.

TABLE XIII.

Agglutination of Claiborn Contact Streptococcus Strains with Scarlatinal Antistreptococcic Sera.

Claiborn contact strains.	Scarlatinal antistreptococcic sera.				
	15a.	15b.	25.	53a.	76a.
C1	—	—	—	—	—
C7	—	—	—	—	+
C8	—	—	—	—	—
C9	++++++	++++++	—	+++	++++++
C10	++	—	—	—	—
C13	+++	+++	+++	++++++	++++++
C15	++++	+++	—	+	—
C18	—	—	—	—	—
C19	—	—	—	—	++++
C20	—	—	—	—	—
C21	—	—	—	—	—
C22	—	—	—	—	—
C23	—	—	—	—	—
C30	—	—	—	—	—
C33	—	—	—	—	—
C34	++++++	++++	+	—	—

Table XIV shows Strain C40 to be closely related to Strain C13, isolated 2 weeks previously from the throat of the patient's brother, and to Strain 76, isolated from one of the cases of scarlet fever which developed at Claiborn Cottage. The failure of other scarlatinal antistreptococcic sera tested to agglutinate Strain C40 is an irregularity which cannot be satisfactorily explained at the present time.

A second study of contacts was made in connection with a case of scarlet fever developing among the women students of the Johns Hopkins Medical School. Immediately after the development of

scarlet fever by the individual mentioned, throat cultures were taken of all the women students frequenting the club hall in which the patient lived, as well as of four men students working in close proximity to her in the anatomy dissecting room. The throats of fifty-

TABLE XIV.

Agglutination of Possible Scarlatinal Contact Streptococcus Strain, No. C40, with Scarlatinal Antistreptococcic Sera.

Sera.	Dilution.					
	1:20	1:40	1:80	1:160	1:320	1:640
15a	—	—	—	—	—	—
15b	—	—	—	—	—	—
25	—	—	—	—	—	—
53a	—	—	—	—	—	—
53b	—	—	—	—	—	—
76a	++	++	++	++	+	+
76b	+++	+++	+++	++	++	+
C13	++++	++++	++++	++++	++++	++++

TABLE XV.

Agglutination of a Series of Scarlatinal Contact Streptococcus Strains with Scarlatinal Antistreptococcic Sera.

Contact streptococcus strains.	Scarlatinal antistreptococcic sera.			
	Sm.15a.	Sm.25.	Sm.53a.	Sm.76a.
D1	—	—	—	—
D2	—	—	—	—
D3	—	—	—	—
D4	—	—	—	—
D5	—	—	—	—
D6	—	—	—	—
D7	—	—	—	—
D8	—	—	—	—
D9	—	—	—	—
D10	++++++	+++	—	++++++

three persons were cultured, thirty-five of whom had been in close contact with the patient shortly before she developed scarlet fever. Of the total number of throats cultured ten showed hemolytic streptococci. The results of agglutination tests of these ten strains are recorded in Table XV.

From but one individual in the series was a hemolytic streptococcus isolated having agglutinating properties similar to those observed among streptococci isolated from scarlet fever. None of the contacts subsequently developed scarlet fever.

SUMMARY.

1. Hemolytic streptococcus has been found in 100 per cent of the throats of patients with scarlet fever during the 1st week of the disease.

2. The average length of time that these organisms are present in the throat varies from 10 to 20 days.

3. No morphological or cultural characteristics peculiar to the hemolytic streptococcus from scarlet fever can be demonstrated.

4. Ten immune sera have been prepared from different strains of scarlet fever streptococci and each of the sera agglutinated more than 80 per cent of the strains isolated from scarlatinal throats. On the other hand, scarlatinal streptococci are not agglutinated by immune sera prepared from hemolytic streptococci isolated from other pathological sources.

5. Serum from patients convalescent from scarlet fever agglutinates weakly or not at all the homologous strain of hemolytic streptococcus.

6. The specificity of the agglutination reaction of scarlatinal streptococci is confirmed by absorption experiments.

7. Scarlatinal antistreptococcic serum affords some degree of protection against virulent scarlet fever streptococci but has no protective power against hemolytic streptococci from other diseases.

8. In a small epidemic of scarlet fever a healthy carrier of hemolytic streptococcus was detected; the organism carried was identical in its serological reactions with strains of hemolytic streptococci isolated from active cases of scarlet fever.

9. In a study of a number of contacts with a case of scarlet fever, in only one instance was a scarlatinal type of hemolytic streptococcus recovered from the throat.

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STUDIES ON ENDOTHELIAL REACTIONS.

VI. THE ENDOTHELIAL RESPONSE IN EXPERIMENTAL TUBERCULOUS MENINGOENCEPHALITIS.

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PLATES 54 TO 56.

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INTRODUCTION.

As stated in the fourth paper of this series, it has been the writer's purpose to call attention to the importance of the vascular endothelium as a source of the mononuclear phagocytes, or macrophages, in conditions resulting from inflammation or injury. Three of the five preceding papers dealt with these phenomena in the case of tuberculous inflammation in rabbits—one with subcutaneous tubercles, one with pulmonary tuberculosis, and one with general miliary tuberculosis (Foot, 1920, *a*, *b*, 1921). In the present paper the reaction of the cerebral tissues to an infection with tubercle bacilli will be discussed. This subject has been reserved for special treatment because of that radical difference between cerebral and other tissues of the body, which might perhaps alter the type of reaction.

There is little doubt that tuberculous meningoencephalitis is a process practically limited to the meninges and their vascular extensions into the substance of the brain. This view appears to be accepted by those few who have written on the subject, upon which little work has been done during the past decade. It is difficult to find a detailed description of the histology of the cerebral lesions in this disease in most text-books on pathology, for the reason that they are to be considered usually a part of a general miliary tuberculosis. Cerebral tuberculosis has, however, some characteristics that differ

rather strikingly from the tuberculosis of other organs. It shows a well marked tendency to limit itself to the mesodermal elements of the brain, a far greater lymphocytic reaction, many large mononuclear cells circulating in the vessels of the affected area, and sometimes fewer giant cells are found here than in tubercles of other organs.

Perhaps the most detailed treatment of the process is to be found in Ranke's (1908) article, in which he gives an excellent historical sketch of the development of knowledge on this subject, tracing it from 1829 when it was first described by Abercrombie. Experimental work was not begun until Kure, working in Nissl's laboratory in 1898, produced tuberculous meningoencephalitis by drawing cotton threads impregnated with tubercle bacilli through the brains of dogs. This method elicited a mixed reaction in which there was, at first, a foreign body to be dealt with and later the infection resulting from the multiplication of the bacilli which had to be overcome. Delille improved on these experiments by injecting extracts of tubercle bacilli in ether, chloroform, and xylol. The tubercles produced in this way were found to be independent of the brain tissue, except where they encroached directly upon its substance.

Ranke's article concludes with a description of three cases of tuberculous meningitis with necropsy and a very careful report on the histopathological examination of the lesions, replete with morphological details based upon the study of preparations stained in a great variety of ways. In the course of this description he discusses large mononuclear cells of varying morphology that are first noted free in the blood of the pial vessels. These cells were first discussed by Tigges in 1863 and interpreted as exfoliated endothelial cells by Sawada in 1901, Diamond taking a similar view as to their origin at about the same time. Ranke, after describing them exhaustively and with the greatest minuteness of detail, leaves the question of their origin open for further investigation and future decision. This is particularly interesting as Farrar (1908), in an article published in the same volume and issued from the same laboratory, describes similar cells in connection with experiments on the reaction to elder pith discs inserted into the cerebral cortex of rabbits, ascribing their origin to the pial endothelium and also tracing the grid cells from the vascular endothelium of the meninges.

Gehry (1909), writing in the following year, takes up the question of the origin of these macrophages and, after discussing them at length, categorically states that they are derivatives of the fixed tissues. As this term is loosely employed in the German literature, it may mean the vascular endothelium, the adventitial connective tissue, or the mesothelium. All these writers are agreed that the brain tissue proper does not respond to foreign bodies, nor to tuberculous inflammation, except secondarily—when it is invaded by the agent in question and by the mesodermal reaction to its presence. In such areas the nerve cells die off, to be replaced by the multiplication of their satellite glia cells, which act as mere stop-gaps. The glia cells form clusters, or nests, and multiply usually by amitotic

division, although mitotic figures are sometimes found. Rod cells are found in the neighborhood of tuberculous lesions in man; the matter of their origin is fully discussed by Uyematsu (1920) in an article on diffuse cerebral sclerosis. He believes them to be modified glia cells, while Alzheimer and Nissl have considered them to be endothelial in origin. Farrar (1908) states that they do not occur in the rabbit's brain.

Shorter discussions of tuberculous meningitis will be found in the section on vascular lesions of the brain and cord by Nonne and Luce (1904) in the *Handbuch der pathologischen Anatomie des Nervensystems*. The fact that cerebral tuberculosis is taken up under the heading of vascular diseases speaks for itself. Lugaro, in the same text-book, gives a good description of the glia cell and its manifold variations in type. According to him, it is the phagocyte of the ectodermal brain tissue, usually multiplying by direct division; it may form giant cells, spider cells or astrocytes, and may also phagocytose and replace ganglion cells, etc. In recent years an article by Friedenwald and Greenfeld (1915) brings the subject of cerebral tuberculosis more nearly to date—but their description of the pathological histology of the lesions is rather meager.

From this review it will be seen that the main question left for decision is the origin of the mononuclear macrophages that circulate in the meningeal vessels and through the lymph spaces of the pia-arachnoid in tuberculous meningitis. That the tubercles are formed in, or near vessels and that their epithelioid cells are probably of endothelial origin has been fairly well established. In order to decide this point, if possible, the following experiment was performed.

EXPERIMENTAL.

Five adult rabbits were anesthetized in turn and a 0.5 cm. opening trephined in the right parietal bone at a point midway between the coronal and lambdoid sutures, about 1 cm. to the right of the sagittal suture. A few drops of a suspension containing in all 0.2 mg. of a 1 month culture of bovine tubercle bacilli on glycerol agar were then injected into the right hemisphere through a 24 gauge needle 1 cm. in length. The same strain of bacilli used in the preceding experiments of the series was employed here. The suspension was prepared as before; a weighed amount of bacilli was rubbed up in sterile salt solution and, after thorough shaking, diluted until 1 cc. equalled 1 mg. of bacteria. Of this suspension 0.2 cc. was injected through the needle, the latter being held as nearly vertical as possible, and

inserted to its full length, releasing the bacilli at the same depth in each brain. There was some leakage from the needle stab after withdrawing the instrument, but this was not without its advantages. The wound was then closed without replacing the bone disc.

All the rabbits were given 10 cc. of 1 per cent aqueous Niagara blue (2 B recrystallized), intraperitoneally, and 5 cc. of Higgins' water-proof drawing ink and distilled water, 50 per cent, intravenously. These dyes were administered thereafter twice a week, until the rabbits became too sick for further injections.

One rabbit became sluggish and lost its appetite at the end of the 2nd week and was killed on the 14th day by injecting Zenker's solution into the right common carotid, under full anesthesia. Nothing was found on gross examination at necropsy, but the use of Niagara blue produces such an intense bluing of the tissues that an early general miliary tuberculosis was overlooked. The other four rabbits became very sick 5 days later, the 19th of the experiment; all were weak and apathetic and all but one had temperatures of 102–102.6°F. by rectum, that one having but 99.9°. The next day the rabbit with the lowest temperature was found dead, after paralysis in its fore legs. It showed an early general miliary tuberculosis and the lower lobe of the right lung was almost completely consolidated and covered with a fibrinous exudate, later ascertained to be an almost complete infarction. The brain showed suspicious sandy nodules about the site of injection of the tubercle bacilli.

The third rabbit was killed the same day, by injecting Zenker's solution as before. It had a definite right-sided weakness for 2 or 3 days preceding death. The brain showed a well developed basal tuberculous meningitis and extensive tuberculous involvement of the brain substance around the injection wound, with edema and swelling of the right hemisphere and right choroid plexus. This was the only brain that showed much gross staining with the Niagara blue, which was confined to the meninges. The internal organs, although they showed a miliary tuberculosis under the microscope, were grossly negative.

The fourth rabbit became progressively weaker, and developed a somewhat inconspicuous left-sided paresis. On the 23rd day of the experiment it was killed in the same manner as were the others; necropsy showed basal meningeal tuberculosis, tubercles along the needle wound, and general miliary tuberculosis.

The fifth rabbit, a strong buck, developed a tendency to turn to the left on the 21st day. After becoming very weak it was killed on the 26th day of the experiment, by an injection of Zenker's fluid into the right common carotid, under ether. At necropsy the findings were similar to those of the preceding four, except that there was more edema of the right temporal lobe near the base.

It is interesting to note the failure of the blue stain to take in any of the brains, with one exception, in which the meninges were distinctly blue. The lesions were not grossly dyed.

In all cases the brain, liver, lungs, spleen, and kidneys were hardened in Zenker's fluid, embedded in paraffin, and cut to 5 microns. Sections were stained with eosin-methylene blue, Mayer's aqueous carmalum, phosphotungstic acid-hematoxylin, and Ziehl-Neelson carbolfuchsin, with Delafield's hematoxylin as a counterstain.

Microscopic Examination.

Each rabbit shows a tuberculous meningitis, the interlobar meninges and choroid plexus are also involved, the vessels running into the brain from the meninges are surrounded near their origin by tubercles and, deeper in the cortex, by a dense zone of lymphocytes and plasma cells. The vascular lesions are more marked in some animals than in others, according to the severity of the infection. The needle puncture is filled with masses of debris, polymorphonuclear leucocytes, and caseous material, surrounded by a zone of actively proliferating endothelial cells in close proximity to one another, which penetrate the surrounding nerve tissue in the form of long, ray-like extensions. Outside of this tuberculous tissue is a zone of degenerated nerve tissue, in which one finds edema, many polymorphonuclears and altered glia cells, and the remnants of nerve cells, but no ink-bearing endothelial macrophages.

In general all the tubercles are more or less deeply pigmented with ink granules; there is little or no Niagara blue, except in the form of coarse granules in the discrete macrophages of the meningeal lymphatics, cells which also contain ink. The changes in the brain tissue are typical and conform to those discussed in the Introduction: degeneration of nerve cells, increase in the number of glia cells, without evidence of mitosis, and polymorphonuclear infiltration of the most affected regions. There are often glia cell nests, or *nebulæ*; the satellite, or *trabant* cells fill the spaces left by the death of the ganglion cells. Apparently the glia cells perform those functions that are elsewhere effected by the endothelial cells, for ink-bearing macrophages are not found at any distance from the tubercles.

The free mononuclear cells discussed so extensively by Ranke and Gehry are found in large numbers in the veins and capillaries of the meninges, less frequently in the arteries. Fully a third to a half of their number contain ink granules, which indicates their endothelial

origin. These cells are frequently found in mitosis while free in the lumen of the blood vessel; three such figures were encountered in the course of half an hour's search, while counting ink-bearing macrophages (Fig. 1).

There is no need for a detailed description of the lesions; that they contain ink, wherever they may be situated, is the main fact to emphasize; hence they are composed chiefly of cells of endothelial origin (Figs. 2 and 3). The reasons for considering any ink-bearing cell as endothelial already have been discussed sufficiently in the preceding papers. Thus the cerebral tubercle conforms in its histogenesis to tuberculosis of other organs. The more interesting point for consideration is the question as to the locus of origin of these endothelial cells. In this case the local activity of the capillary endothelium in the neighborhood of the lesions is not nearly as marked as it was in the lesions of other organs studied in the preceding experiments. Thickening of the capillary endothelium and an increase in the number of its nuclei are evident here and there, especially where the brain tissue has been more extensively destroyed, but on the whole these changes are not at all marked, nor are mitoses found to occur in the sessile endothelial cells of these vessels. It will be recalled that these lining cells were frequently found in mitosis in the other experiments and that this usually occurred near the lesions.

Ink is not found in the ependymal cells of the ventricles or choroid plexus (Fig. 4). Where tubercles occur, they are always beneath the ependyma and covered by it, and they contain the usual amount of ink. The ependymal cells are, therefore, very similar, in their lack of response to the tubercle bacilli and injected pigments, to the mesodermal cells of the peritoneum. It was always the subperitoneal, perivascular tissue that responded in the cases of tuberculous peritonitis studied in the previous experiments.

Whence, then, come the endothelial cells that go to form the numerous tubercles in these brains? Ranke and Gehry called attention to circulating mononuclears. These cells are very abundant in all five of the rabbits studied; furthermore, many of them are fixed in the act of mitosis, proving that they are actively proliferating. Much has been written about them, little definitely proved; they probably come from other organs more richly supplied with capillaries than

is the brain. Mallory has long maintained the endothelial origin of circulating mononuclears and more recently McJunkin (1919) has reached a similar conclusion. Downey (1916-17), Evans (1915), and Simpson (1922) suggest that they proceed largely from the reticuloendothelium of the hematopoietic organs, particularly the spleen. A glance at the sections from the spleen (Fig. 5) and the liver (Fig. 6) of the animals of this experimental series is sufficient to make it clear that pigmented endothelial cells are leaving the capillary walls and entering the veins, to be thrown into the circulation in numbers somewhat larger than would be the case following the mere injection of ink (Simpson's "shower of macrophages"). In the lung they are also found in the larger vessels, and the pulmonary endothelium is rather swollen and active in places, but it must be remembered that here, as well as in the other organs, an active miliary tuberculosis is in progress. Although not much increased in number over what one finds in control animals without infection, they show a process not seen in controls—the phenomenon of mitotic division while free in the blood stream.

Supplementary Experiment.—In order to rule out at least one of the hematopoietic organs as the sole source of these circulating macrophages, the spleen was removed from four rabbits, which were then inoculated with tubercle bacilli of the same strain and in the same manner as the preceding series of animals. The cultures were younger (2 weeks) so that a smaller dose was administered, in order to decrease the toxicity. Only 0.16 mg. was given in these cases. Following the operation, which was performed under ether anesthesia, 5 cc. of Higgins' ink and distilled water in equal parts were injected into an ear vein 1, 5, and 8 days afterwards; the dose was repeated in two of the rabbits on the 12th day. These two died almost immediately with symptoms suggesting cerebral embolism. Necropsy showed no gross lesions, beyond an unusually light-colored liver and almost jet-black lungs. Microscopic examination showed that death was due to cerebral emboli of ink, which were scattered all through the precapillaries of the cortex and were plugged quite solid with the ink. There were occasional ink emboli in the lungs, but not sufficient to give death from this cause. The remaining two rabbits went uninjected for 2 weeks, when another intravenous administration of 5 cc. of the

ink mixture was given, this time uneventfully. Another injection, 1 week later, killed the third rabbit in precisely the same way the other two had been overwhelmed and necropsy gave similar findings. The fourth rabbit was allowed to go uninjected for 4 weeks and was then killed by introducing Zenker's fluid into the right common carotid under anesthesia.

The glass pestle used in triturating the bacilli for the suspension injected into these splenectomized animals apparently was not sufficiently cooled after flaming, for the bacilli were killed. Very good local reactions (Hodenpyl tubercles) were, however, obtained; they differed from those of the preceding experiment merely in so far as they were localized, did not spread to other organs, and were not accompanied by generalized symptoms. Despite the fact that the spleen had been removed, the tubercles were formed and the macrophages were found in apparently undiminished numbers lying free in the meningeal vessels. An examination of the liver and bone marrow revealed no increased production of macrophages, indeed they seemed to contain less ink than in the case of control animals, and there was no undue activity of their endothelial elements. The lungs, on the other hand, showed changes so striking that they call for another experiment to determine their cause and to follow the course of their development. This will be described in another article. The proliferation of the pulmonary endothelium, the swelling of its component cells, and the increased amount of contained ink in the organ are most remarkable.

We may conclude, then, that cerebral tubercles are produced somewhat differently from those of other organs, in that the endothelial cells that form them are evidently derived only in part from the local capillaries; the circulating macrophages showing much more proliferative activity than the sessile endothelial cells of these capillaries probably form a more important source of supply. Hence it appears that a much greater proportion of the endothelial cells of cerebral tubercles is derived from the circulating blood than is the case in tubercles of other organs. This conforms with the older French theories on the histogenesis of the tubercle.

CONCLUSIONS.

1. Experimental cerebral and meningeal tubercles in the rabbit are formed from cells of endothelial origin.
2. These cells are derived apparently from other sources than the neighboring capillary endothelium alone.
3. The circulating macrophages, which, in this case, are capable of multiplying by mitosis while still free in the blood, are drawn upon in the formation of the cerebral tubercle.
4. Splenectomy has not materially decreased the available supply of circulating macrophages in this experiment.
5. While these cells may originate in the endothelium of the liver and bone marrow, the lung appears to play a much more important rôle in this respect than has been hitherto suspected.

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EXPLANATION OF PLATES.

PLATE 54.

FIG. 1. Macrophages circulating in a meningeal vessel. Some are clumped, others discrete; one of these cells is seen in mitosis at the center of the photograph. $\times 400$.

FIG. 2. Cerebral tubercle. The ink granules are so coarse that most of them are readily confused with nuclear material in the photograph. Several of the epithelioid cells near the periphery show finer granules. $\times 335$.

PLATE 55.

FIG. 3. A meningeal tubercle. The ink shows to better advantage. $\times 335$.

FIG. 4. Tubercle in the choroid plexus. Note that carbon is not seen in the ependymal layer, which remains aloof from the process going on beneath it. $\times 335$.

PLATE 56.

FIG. 5. Group of macrophages leaving the spleen, one of them in mitosis, most of them heavily laden with ink. $\times 335$.

FIG. 6. Macrophages in an hepatic vein and in the sinusoids of the liver, in one place clumped into a mass. $\times 335$.

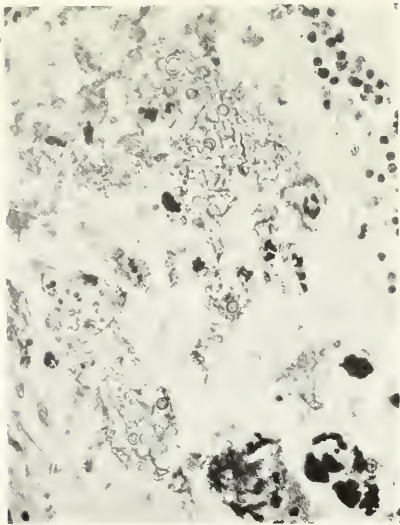


FIG. 1.

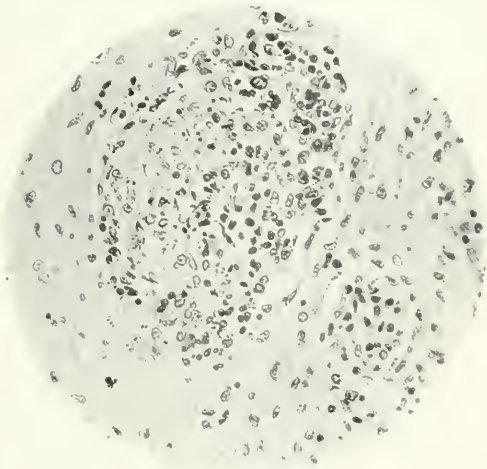


FIG. 2.

(Foot: Endothelial reactions. V1.)

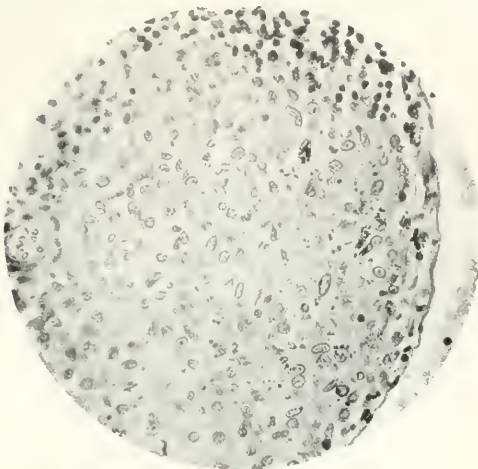


FIG. 3.

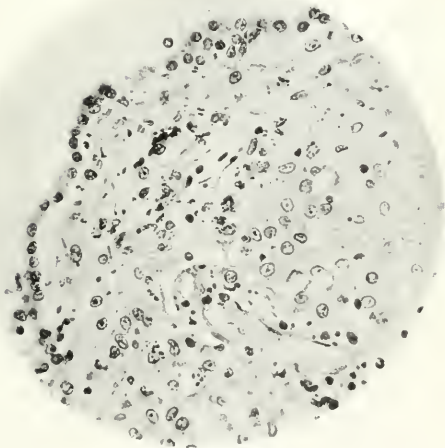


FIG. 4.

(Foot: Endothelial reactions. VI)

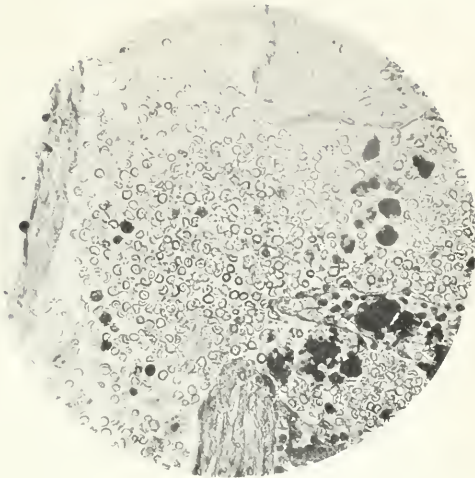


FIG. 5.

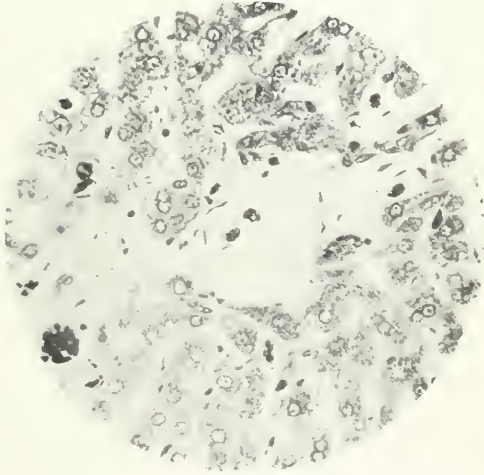


FIG. 6.

(Foot: Endothelial reactions. VI.)

SPIROCHÆTA EURYGyrATA.

A NOTE ON ITS LIFE HISTORY AND CULTIVATION.

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PLATE 57.

(Received for publication, June 24, 1922.)

For some time it has been known that spirochetes are occasionally present in the intestine of man.

In 1894 Kowalski (1) mentioned finding large numbers of spirochetes in the stools of patients suffering with cholera, and said he had seen them in the cholera epidemic in Paris in 1884. In 1903 Le Dantec (2) found almost pure cultures of spirochetes in the dysenteric stools of a patient in Southwest France. He believed them to be the cause of the dysentery. In 1907 Mühlens (3) reported finding small spirochetes with two to four spirals in a case of amebic dysentery, in Southwest Africa. He described them as active and as having pointed ends. He found similar organisms in children suffering with summer diarrhea and also in cholera stools. He considers them non-pathogenic.

Werner (4) in 1909 described two types of spirochetes which he found in his own stool after typhoid fever: one which he calls *Spirochæta eurygyrata*, loosely coiled, very active, and flexible, with rarely more than two spirals, mostly an S form; the other type, *Spirochæta stenogyrata*, tightly coiled, not so active, and less flexible. He finds it hard to compare these types with Le Dantec's spirochetes on account of the latter's meager description, but concludes that his first type corresponds to Le Dantec's. He found Type I in an Arab who had no intestinal disturbances, and also in a case of chronic diarrhea, and found both types in three cases of sprue. He concludes that it is impossible to say whether or not the spirochetes are pathogenic.

In 1914 Thomson and Thomson (5) also reported finding spirochetes in the alimentary tract of man and thought they corresponded to Le Dantec's. These measured 8 to 20 μ . They had rather blunt ends, large irregular curves, and showed a great flexibility of the body.

In 1916 army medical officers began reporting the presence of *Spirochæta eurygyrata* as an intestinal parasite. Fantham (6) described it as having pointed

ends, and being 3 to 15 μ in length. He believed that the number of coils is not a specific character, but is variable, depending on the thickness of the organism. Thomson and Thomson (7) list seven varieties of spirochetes frequently found in feces, especially in diarrheal condition. Macfie (8), at autopsies, showed spirochetes to be present in the stomach, at various parts of the small intestine, in the cecum, and in the large intestine. They appeared to him to be non-pathogenic, but he concludes that this question cannot be considered settled.

Luger (9) found *Spirochæta eurygyrata* in thin, often bloody, stools. He believed that the spirochetes can appear in varying numbers in the intestine, as normal parasites, but under certain conditions, not well understood, they may lead to a serious injury of the intestine, the therapy of which is little known. In 1918 Porter (10) described them as being 15 μ long with tapering ends. She believed that their presence in large numbers is indicative of intestinal disorder. Recently Langendörfer and Peters (11) described the case of a soldier who had a spirochetal infection accompanied by bloody stools. The spirochetes had six to eight tight spirals. They were motile, but not flexible. The Wassermann reaction was negative. The patient was treated with salvarsan, and in 3 months was well. These authors were not sure that the spirochetes were the cause of the dysentery, but were certain that they were present and caused the bloody condition.

It will be seen from the foregoing résumé that there is a great variation in the descriptions by different writers of the organisms which many of them call *Spirochæta eurygyrata*, and also in their conception of its pathogenicity. It was with the hope of reconciling some of these rather contradictory reports that the present work was begun.

The material from which this study has been made was sent to the laboratory, January 26, 1921. Loopfuls of the liquid feces, containing *Spirochæta eurygyrata*, were transferred to culture media and the organism was cultivated. On March 2, two pure lines, Strains S1 and S2, were isolated. These pure lines were kept in culture until June 6, when they had all died. Last fall, November 12, another stool specimen from the same case was procured and organisms from this have been kept in culture and are still flourishing (June, 1922).

History of the Case.—The patient from whom the material was obtained was a woman who had been suffering with chronic diarrhea for 13 years. All attempts to control it have failed. There is a triple infection of *Chilomastix mesnili*, *Trichomonas hominis*, and *Spirochæta eurygyrata*.¹

¹ I am indebted to Dr. Charles Simon for the material and history of the case.

EXPERIMENTAL.

Frequency of Occurrence.—In this part of the country *Spirochæta eurygyrata* does not seem to be of frequent occurrence. A survey was made of 180 stools sent into the laboratories of the Johns Hopkins Hospital from 105 patients, but *Spirochæta eurygyrata* was not found in any of them.

In the eastern hemisphere the reports are quite different. Macfie (8) found all the persons examined in the Gold Coast colony to be infected, and notes that Carter, examining dysenteric soldiers, found 56.5 and 41.5 per cent of the non-dysenteric soldiers infected. Porter (10), who was also working in Africa, found twenty-nine spirochetal infections out of 93 cases examined and says they rank next to *Entamæba coli* infections in frequency of occurrence. Fantham (12) in a survey of 1,305 dysenteric patients, with a total number of 3,325 examinations, found spirochetes only 42 times; while Faust and Wassell (13) examined 359 cases in Wuchang and found 0.3 per cent infected with *Spirochæta eurygyrata*.

Culture Media.—The 1st year, Locke-egg, ovomucoid, and ox bile salts media were used. These had already proved excellent media for the cultivation of *Trichomonas hominis* (Hogue (14)) and *Waskia intestinalis* (Hogue (15)). The 2nd year, tubes containing 15 cc. of sterile 0.85 per cent sodium chloride solution and 0.3 cc. of sterile serum water were used. In the beginning, pig serum, diluted 1:4, and sheep serum, diluted 1:3, were both used, but it was soon found that the spirochetes lived longer and were more active in the medium containing pig serum.

The media used had a pH of 6.8, 7, and 7.4. In each of these the spirochetes grew, though the medium with a pH 7 gave the best results. The tubes were covered with layers of paraffin oil to prevent evaporation and to keep them from contact with the air.

Sterile pieces of rabbit kidney were put into some tubes of this sodium chloride-serum water medium and sealed with vaseline, but in these tubes the spirochetes did not multiply and lived only a short time. They are not anaerobic.

Age of Cultures.—In ovomucoid, Locke-egg, and ox bile salts media the spirochetes usually lived from 8 to 12 days. Transfers from these media were made every 2 or 3 days. In one rare case active spirochetes were found in ox bile salts medium 70 days after inoculation.

In the deep cultures of sodium chloride-serum water, covered with paraffin oil, they lived much longer. Here many cultures had active spirochetes after 46, 62, 64, and three after 94 days. One tube still contains many active spirochetes 127 days after inoculation. In this tube there are both long and short individuals, showing that division is still taking place and that the culture is in good condition. Evidently an equilibrium has been reached which completely satisfies the organisms.

Method. Isolation of Pure Lines.—Loopfuls of the semiliquid stool were put into test-tubes of various media and these were incubated at 35°C. In a few days there was a good growth of spirochetes of all sizes. From these cultures very small hanging drops were made and examined microscopically. When a drop was found which contained only one spirochete, the edges of the cover-slip were cut off and the remaining piece of glass, with its small drop of culture medium, was put into a test-tube with fresh medium. This spirochete was the beginning of a pure line; *i.e.*, a line derived from a single individual. This method was found better than isolation with the Barber pipette, since the spirochetes move very rapidly.

An attempt was made to free the spirochetes from the intestinal bacteria growing with them.

Nine large test-tubes containing 2 per cent agar, 0.7 per cent sodium chloride, and 0.4 per cent peptone were cooled to 50°C. To each tube 0.3 cc. of sheep or pig serum water was added. When hard, these tubes were inoculated with spirochetes by means of a capillary pipette and covered with paraffin oil to prevent drying. They were incubated at 35°C. 18 days later the test-tubes were broken into sterile Petri dishes and the agar cylinder was cut open. In the line of inoculation were many active bacteria and inactive spirochetes. Pieces of agar were cut from the sides of the column of growth and transplanted to tubes of sodium chloride-serum water. When these were later examined, no spirochetes were present. It is a question whether the supply of oxygen was insufficient for their maintenance, or whether they were unable to migrate into this dense medium.

Stains.—The spirochetes were stained with iron-hematoxylin, Giemsa's stain, Cross' stain (16), and weak carbolfuchsin. A loop of the culture containing a large number of the spirochetes was smeared on a cover-glass. This was killed in hot Schaudinn's fluid and then stained in iron-hematoxylin over night. With Giemsa's stain the

cover-slips were fixed in methyl alcohol. Cross' stain gave very good results and was a quick method. The smears on the slides were dried in the air and stained 2 minutes. Carbolfuchsin diluted also gave excellent results.

Vital Stains.—Neutral red, methylene blue, brilliant cresyl blue, Janus green, and pyrrol blue were all used. Varying concentrations of the stain were added to hanging drops of the culture. The cover-slips were sealed with vaseline and the spirochetes kept under observation often for 24 hours, but in no case were the organisms colored by these vital dyes.

Dark-Field Examination.—With dark-field illumination the spirochetes showed no flagella. Both ends were rounded. A dark line ran the entire length of the body in the midline. On either side of it were bright refractile zones. When the spirochetes were alive and active there were no cross-bars, or banding. As they moved a dark wave passed along the body from the anterior to the posterior end. This was only transitory.

In the old cultures in which the spirochetes were no longer active and therefore considered to be dead (Hogue (17)) they presented a very different aspect. The parallel lines of refraction were broken up. There were dark bars which went three-fourths or entirely across the body. The tops of the crests were usually very bright. It seemed as though the cytoplasm had become concentrated at these points.

These cross-bars, seen with dark-field illumination, of spirochetes in culture media, are further evidence for Noguchi's (18) theory "that the cross bands are formed from a homogeneous mass through sudden contraction." From his stained preparations he thinks it is "due to dehydration with absolute alcohol during fixation." In the present case the process is undoubtedly a slower one, taking place at the time of death. At this time the cytoplasm containing the diffuse chromatin material is concentrated at certain places along the body. When these are stained with iron-hematoxylin they take the chromatin stain, and have been interpreted by many as chromatin bars. In reality they are an artifact due to the coagulation of the protoplasm after death.

Effect of Different Media.—There was little morphological difference in the spirochetes grown in the different media. The very long forms

with over twenty crests were found in both pure lines (Strains S1 and S2), in Locke-egg and in ox bile salts media. There may have been some substance in these media which delayed or prevented division.

In the ox bile salts medium a larger number of the spirochetes seemed to be loosely coiled and flexible, and, consequently, to move rather with a snake-like than a corkscrew-like motion.

There was also a noticeable difference in the rate of growth in the media. The greatest number of individuals in a culture of ovomucoid appeared on the 2nd day, of Locke-egg on the 7th day, and of sodium chloride-serum water covered with paraffin oil on the 14th day or even later.

Motion.—The motion of the spirochete depends largely on its size and consequently on its age. The short and medium sized spirochetes are very active. There is the typical spirochete motion, the darting back and forth. As they grow longer this motion becomes impossible and one notices a wave-like, undulating motion which passes the length of the body and often appears as a twisting of the entire body from side to side. The body is not rigid but appears quite flexible, bending at any part. This wavy, almost worm-like, motion is always indicative of age. It becomes less and less until finally all motion ceases. If these organisms are transferred to fresh culture media there is no growth or multiplication, showing that they are dead. Here again motion and locomotion can be taken as a criterion of life, as I (17) have already shown to be the case for some other organisms.

Morphology.—Some former observers have quite rightly said that the length of the spirochete and the number and twist of the spirals cannot be taken as criteria for the determination of different species. The two organisms used to start the two pure lines (Strains S1 and S2) both had three crests. In the races derived from them were subsequently found individuals varying in length from 4μ to 56μ (Figs. 1 and 12). In the young cultures, about a week old, the spirochetes generally have one, two, or three crests (Figs. 1 to 6). These shorter forms increase in size until the very long forms (Figs. 12 and 14) appear in great numbers in cultures 3 or more weeks old. Some of these have as many as twenty-seven crests.

These older forms often have stretched spirals; *i.e.*, places where the coiling is irregular (Figs. 12 and 14). They frequently bend at these places (Fig. 14) while they are twisting from side to side.

There are also in these pure lines both Type I (Fig. 5) and Type II (Fig. 6) of Werner's spirochetes; *i.e.*, those which are loosely coiled and flexible, and those which are more tightly coiled and less flexible. However, I find Type I not so active as Type II, which with its tighter coils has more of the corkscrew motion of *Treponema pallidum*. Type I is also slightly thicker than Type II with its tighter coils.

In the literature there is a great discrepancy as to the kinds of ends which the spirochetes have. In all the spirochetes which were cultured in the present study both ends have been rounded. Occasionally a few of the short forms will have slightly pointed ends (Figs. 2, 3, and 5) but I have never observed sharply pointed ends, or ends drawn out into filaments.

The cross-bars appearing in stained preparations and described by so many observers of spirochetes have already been shown to be artifacts due to the coagulation of the protoplasm which contains diffuse chromatin. The dyes used were all basic and consequently stained the chromatin material. These bars were not very wide, nor were they of uniform width (Fig. 5). As a rule they were not arranged at regular intervals, though they were usually at the crests (Figs. 3, 5, and 6). In some cases the dark areas did not extend entirely across the spirochete (Fig. 4). This may be due to too much differentiation of the stain, but more probably the material was coagulated at one side of the organism, as appeared to be the case with dark-field illumination. One also notices in many of these stained preparations a massing of this deeply staining material at one or at both ends of the organism (Figs. 3 to 5 and 7). With weak carbolfuchsin and with Giemsa's stain the bars were visible, but were not nearly so distinct. With Cross' stain the whole organism was usually stained a deep purple, though, with shorter exposure to the stain, faint bars could be distinguished along the body.

Reproduction.—Transverse division is the only kind of reproduction which has been so far observed for *Spirochæta caryogyrata*. It may be equal or unequal. In the shorter forms it is usually equal (Figs. 7 and 9). In the longer forms it may be equal (Fig. 15), but is more

likely to be unequal (Figs. 8 and 11 to 13). Indeed it may be so unequal that it can almost be called fragmentation (Fig. 12). Some individuals were found which, after being stained with Cross' stain, showed several clear spaces (Fig. 16). These forms would probably fragment into several small individuals. This mode of reproduction, no doubt, accounts for the numerous short forms seen in the cultures a week old, which have been started by inoculating tubes from older cultures full of long forms.

Incurvation was occasionally seen (Fig. 10) and was interpreted as transverse division, the spirochete being twisted upon itself in its effort to separate into two individuals.

Porter (10) has described coccoid bodies which are formed and serve as a means of transmitting the organism. In the older cultures there are certain individuals which seem to contain one or more refractile granules, situated usually in the crests. With dark-field illumination these bodies were interpreted as being the concentration of the protoplasm due to death. They were never seen in active spirochetes in the dark field. Moreover, they have never been seen as separate bodies in the culture medium, nor have they been found with the young spirochete growing from them as has been so often described for other spirochetes.

Animal Experimentation.—It was hoped that rats could be used as hosts for these spirochetes, but after making daily fecal examinations of four rats for 8 days, it was found that two of them were already infected with a spirochete which appeared at irregular intervals in the stools. Other rats were examined and found infected, so it was decided not to use this animal.

Several cats were found infected with the spirochetes described by Kasai and Kobayashi (19), but finally by daily fecal examinations for 8 days three were found which were not infected. These were each fed 6 cc. of a culture of active *Spirochæta eurygyrata*. On 8 succeeding days their stools were examined microscopically and culturally but no spirochetes were found. 43 days later the cats were again examined but still with negative results. It seems evident that the cat cannot become infected through the mouth with this species of spirochete.

SUMMARY.

1. Pure lines of *Spirochæta eurygyrata* were isolated and cultured.
2. They lived longest in a medium which was made up of sodium chloride and pig serum water.
3. They stained readily with iron-hematoxylin, Giemsa's stain, Cross' stain, and carbolfuchsin.
4. They did not stain with the vital dyes used.
5. They divide by transverse division.
6. Transverse bars, which are seen in stained spirochetes and in dead ones with dark-field illumination, are considered coagulated protoplasm, due to death.
7. Coccoid bodies were not seen in living individuals nor were they seen free in the culture medium.
8. Cats were fed cultures of *Spirochæta eurygyrata* but did not become infected with them.
9. *Spirochæta eurygyrata* is not of frequent occurrence in this part of the United States.

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EXPLANATION OF PLATE 57.

All the drawings were made with a camera lucida. Magnification, $\times 3,000$.

FIG. 1. A short *Spirochæta eurygyrata* stained with carbolfuchsin.

FIG. 2. A short form with pointed ends, stained with Cross' stain.

FIG. 3. A short form stained with iron-hematoxylin to show the cross-bars.

FIG. 4. A short form stained with iron-hematoxylin. The bars do not extend entirely across the body.

FIGS. 5 and 6. Short forms showing the cross-bars extending across the body. Stained with iron-hematoxylin.

FIG. 7. A short form preparing for equal transverse division. There is a massing of the deeply staining material at each end. Stained with iron-hematoxylin.

FIG. 8. A longer form dividing by unequal transverse division. Cross' stain.

FIG. 9. A short form dividing by equal transverse division. Stained with carbolfuchsin.

FIG. 10. Incurvation, interpreted as transverse division. Cross' stain.

FIG. 11. A form dividing by unequal transverse division. Stained with carbolfuchsin.

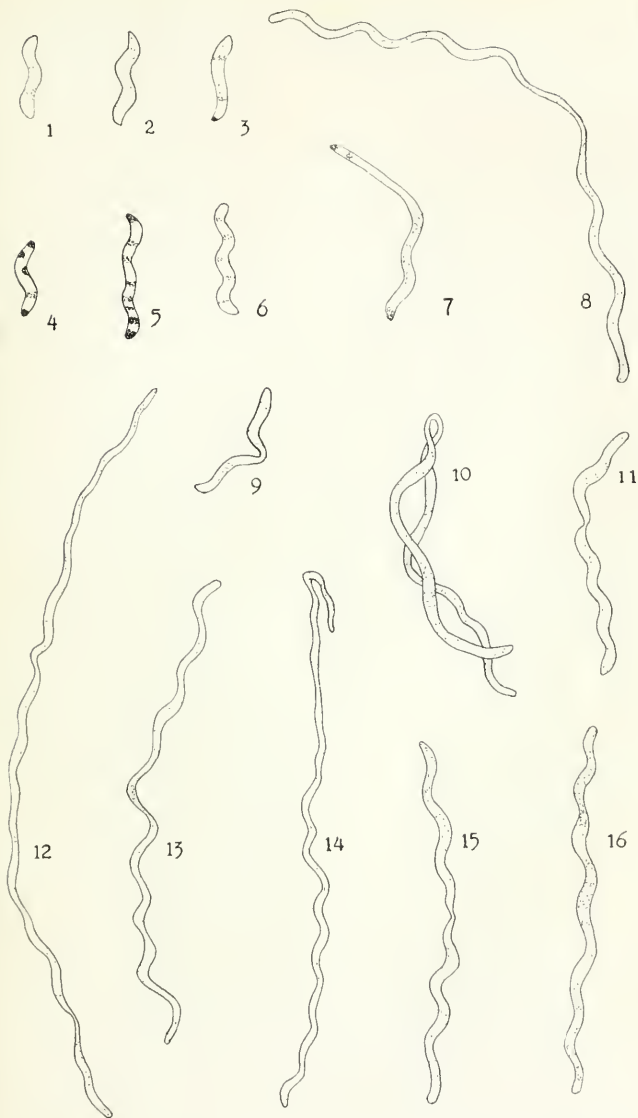
FIG. 12. A long form dividing by very unequal division. Cross' stain.

FIG. 13. A long form dividing by slightly unequal division. Cross' stain.

FIG. 14. The bending of the spirochete at the place where unequal division is taking place. Iron-hematoxylin stain.

FIG. 15. A spirochete dividing by equal transverse division. Cross' stain.

FIG. 16. A spirochete preparing to divide into six smaller individuals. Cross' stain.



(Hogue: *Spirochaeta euryspirata*.)

INSUSCEPTIBILITY TO SENSITIZATION AND ANAPHYLACTIC SHOCK.

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(Received for publication, July 10, 1922.)

The object of the experiments that are to be described was to gain some information concerning the factors which determine the variations that species and individuals show towards certain forms of intoxication. The study forms a continuation of some investigations previously made with Mackenzie (1) and later completed by Mackenzie and Leake (2). In these previous investigations the immunological processes involved in the production of serum disease in the human being were selected as the problem.

It is well known that the incidence of serum disease following the injection of therapeutic sera increases roughly in proportion to the amount of serum injected, so that up to a certain point the susceptibility to serum disease is dependent upon the size of the dose. After a dose of approximately 75 to 100 cc. of serum is reached the incidence of serum disease remains about stationary at 80 to 90 per cent, and no matter how much more serum is administered, a small proportion of individuals always escapes the manifestations of serum sickness.

Recently, Coca, Deibert, and Menger (3) have shown, moreover, that the North American Indian is relatively much less susceptible than the white race, for they state that of twenty-six healthy Indians each of whom received 100 cc. of normal horse serum intravenously, only twelve, or 46 per cent, developed symptoms of serum sickness, and in most of these instances the disease ran a short and very mild course, averaging only 2 days.

It was desired in the previous experiments to study the protective mechanism possessed by the small group of white patients that escaped serum sickness. These individuals were found to differ from the susceptible inasmuch as they did not produce demonstrable antibodies in the form of precipitins and anaphylactic antibody, and inas-

much as the antigen, or horse serum, remained in the circulation as an apparently innocuous substance for periods of weeks or months after it was injected. In the susceptible individuals precipitins were usually formed in large amounts, and the antigen disappeared rapidly from the circulation with the subsidence of serum disease. It seemed, therefore, that the insusceptible individual possessed some mechanism which inhibited the union of antigen with the cells of the body and thus prevented the effects which follow the rapid and usual reactions between the foreign protein and the tissues of the host. According to Metchnikoff, a similar form of mechanism explains the insusceptibility of certain cold blooded animals to diphtheria and tetanus toxin. And according to Coca, Russell, and Baughman (4) the partial impermeability of the cells of the rat to diphtheria toxin explains their great resistance to this poison.

Individual variation to sensitization by foreign proteins is known to occur in all laboratory animals but in the guinea pig or even the rabbit, it is so rare that experiments with these animals seemed unprofitable.

The white rat, on the other hand, has generally been considered as an animal which could not be made anaphylactic (Doerr (5)). It is not possible, however, to find many observations in the literature which afford much information on this subject.

Arthus (6) simply states that he has observed anaphylactic phenomena in white rats after repeated injections of foreign sera. Rosenau and Anderson (7) mentioned the fact that they had tested the anaphylactic reaction in monkeys, rabbits, mice, dogs, cats, rats, chickens, and pigeons and stated that they obtained positive results with the dog, rabbit, and cat. Uhlenhuth, Haendel, and Steffenhagen (8) reported that they were unable to produce anaphylactic shock in white rats or mice. These observations were confirmed by Trommsdorff (9), who failed in attempts to sensitize twenty-five white rats and twenty-five mice to egg white or to horse serum. The second injection of these proteins was administered intravenously after intervals of 5 days to 12 weeks. But the most complete study of this subject has probably been made by Novy and De Kruif (10), who, in attempting to sensitize white rats to horse serum, beef serum, rabbit serum, and egg white, found that the second injection made intravenously of such large amounts of the undiluted antigens as 2 to 4 cc. in these supposedly sensitized rats did not produce any symptoms which could definitely be considered as anaphylactic, and was never fatal. When, on the other hand, antigens diluted with distilled water were given intravenously in amounts of

7 to 10 cc. at the second injection, violent symptoms occurred and in many animals were followed by death. In the normal rat similar amounts of the mixture of antigen and distilled water produced little untoward effect. The intoxication brought about in the supposedly sensitized rat, by the second injection of diluted antigen, proved, however, to be non-specific, for it could be produced quite as readily by the injection of an equal amount of distilled water without antigen. Novy and De Kruif concluded, therefore, that though the rat was practically insusceptible to specific anaphylactic shock, the repeated injection of a foreign protein, nevertheless, produced some change which rendered the animal highly sensitive to the intravenous injection of distilled water and gave rise to a state which was conducive as well to a much more rapid formation of anaphylatoxin in the serum of the shed blood than could be obtained with normal rat serum. Novy and De Kruif (11) also studied the effect upon white rats of the intravenous injection of anaphylatoxin, agar sol-gel, and peptone. To all of these substances the rats were much more refractory than guinea pigs. Weight for weight, the rat tolerated 100 times as much anaphylatoxin as guinea pigs, three times as much agar sol-gel, and about seven times as much 10 per cent peptone solution. For white rats the lethal dose of 10 per cent solution of Witte's peptone was 2 gm. per kilo.

From these experiments it seems evident that the white rat is both highly refractory to anaphylaxis and very resistant to the action of such toxic substances as anaphylatoxin and peptone.

EXPERIMENTAL.

In the present investigation the following plan of procedure was adopted: (1) study of the symptoms produced in white rats by intravenous injection of peptone and histamine; (2) attempts to sensitize white rats to horse serum; (3) determination of the state of sensitization (*a*) by intravenous and subdural injection of antigen, (*b*) by skin reactions, and (*c*) by the uterine strip reaction; (4) attempts to sensitize passively guinea pigs with the serum of white rats immunized to horse serum; and (5) study of the antigen-antibody content of the serum of white rats injected with horse serum.

Symptoms Produced by the Intravenous Injection of Peptone and Histamine.

In order to obtain some criteria of shock similar to that in anaphylaxis, white rats weighing approximately 100 to 125 gm. were given intravenous injections of solutions of Witte's peptone and of histamine.

1 gm. of Witte's peptone was dissolved by heat in distilled water, centrifuged, and the supernatant fluid employed for injection. The histamine was made up in 1 and 2 per cent solution in 0.85 per cent NaCl.

The intravenous injection of 0.1 gm. of peptone per 100 gm. of body weight caused very severe symptoms. Immediately after the injection the animals went into collapse. They lay flat upon the table with all four legs spread out; the respirations were slow and difficult. After 2 to 3 minutes they often made an attempt to stand but failed; within 4 or 5 minutes there were sometimes slight convulsive movements with irregular gasping respirations; in about 10 minutes the respirations would become rapid (68 in one animal), while the paralysis and spasmodic convulsive movements continued. One animal recovered after this dose at the end of 2 hours. A second animal died in 10 minutes.

The effects following the intravenous injection of histamine were quite similar; immediate collapse, gasping respiration, paralysis, convulsive seizures, fall in temperature, the expulsion of feces and urine, and the discharge of fluid from the nose were all prominent features. One noteworthy symptom was an extreme and acute exophthalmos. In those animals that recovered, the severe symptoms persisted for an hour or longer. Two rats withstood a dose of 10 mg. of histamine per 100 gm. of body weight, two animals 15 mg. of histamine per 100 gm. of body weight, and one animal died after receiving 30 mg. of histamine per 100 gm. of body weight.

Attempts to Sensitize to Horse Serum.

A criterion having been established for symptoms which by analogy with other animals should simulate anaphylaxis in the white rat, attempts were made to sensitize white rats by repeated injections of horse serum given subcutaneously, intraperitoneally, intravenously, or subdurally. Twenty-seven experiments in twenty-three rats weighing from 75 to 150 gm. were devised for this purpose. Thirteen rats were given three injections of from 0.1 to 0.5 cc. of horse serum subcutaneously or intraperitoneally at 2 to 3 day intervals, three rats six injections of similar amounts, two rats a single injection of 0.5 cc. intravenously, and five rats three injections subcutaneously or intra-

TABLE I.

Pat. No.	Weight. <i>gm.</i>	Date sensitized. <i>1921</i>	Amounts of horse serum. <i>cc.</i>	Method.	Length of time after last injection. <i>days</i>	Amount of horse serum. <i>cc.</i>	Method.	Symptoms.
1	85	Oct. 5	0.5	Subcutaneous.	21	2.5	Intraperitoneal.	None.
		" 7	0.5	"				
		" 10	0.5	"				
2	84	" 5	0.5	"	22	0.5	Intravenous.	"
		" 7	0.5	"				
		" 10	0.5	"				
4	94	" 6	0.5	Intraperitoneal.	21	0.5	"	"
		" 7	0.5	"				
		" 10	0.5	"				
6	83	" 5	0.5	"	22	0.5	"	"
		" 7	0.5	"				
		" 10	0.5	"	(Jan. 5, 1922.) 66	1.0	"	"
7	154	" 17	0.1	Subcutaneous.	29	0.6	"	"
		" 20	0.25	"				
		" 24	0.25	"				
8	138	" 17	0.1	"	29	0.5	"	No definite symptoms.
		" 20	0.25	"				Remains quiet; respira-
		" 24	0.25	"				tions a trifle irregular.

TABLE I—Continued.

Rat No.	Weight.	Date sensitized.	Amounts of horse serum.	Method.	Length of time after last injection.	Amount of horse serum.	Method.	Symptoms.
	gm.	1921	cc.		days	cc.		
11	136.5	Oct. 17	0.1	Intraperitoneal.	29	0.5	Intravenous.	None.
		" 20	0.25	"				
		" 24	0.25	"				
13	77	" 24	0.2	"	31	1.0	"	"
		" 26	0.4	"				
		" 28	0.5	"				
14	68	" 24	0.2	Subcutaneous.	31	0.5	"	Lies flat 5 to 10 min. after injection.
		" 26	0.4	"				
		" 28	0.5	"				
15	106	" 24	0.2	"	31	0.8	"	Lies flat 5 min. after injection. Seems weak.
		" 26	0.4	"				15 min., recovery.
		" 28	0.5	"				
15	150	Nov. 29	0.8	Intravenous.	38	1.0	"	None.
16	83	Oct. 24	0.25	Intraperitoneal.	31	1.0	"	"
		" 26	0.5	"				
		" 28	0.5	"				
17	93	" 24	0.2	"	31	1.0	"	"
		" 26	0.5	"				
		" 28	0.5	"				

18	89	Oct. 24 " 26 " 28	0.2 0.5 0.5	Intraperitoneal. " "	31	1.0	Intravenous.	None
19	98	Nov. 2	0.5	Intravenous.	21 44	0.5 0.5	" Intraperitoneal.	" "
20	150	" 2	0.5	"	21 44	0.5 0.5	Intravenous. Intraperitoneal.	" "
33	92	¹⁹²² Jan. 5	0.2	"	27	1.0	Intravenous.	"
41	125	" 6 " 9 " 11 " 13 " 16 " 18	0.2 0.5 0.5 0.5 0.5 0.5	Intraperitoneal. " " " " "	27	1.0	"	Seems slightly weak but walks well. No respira- tory difficulty.
Controls, 21	127	" 11 " 13 " 16 " 18	0.5 0.5 0.5 0.5	" " " "	¹⁹²¹ Nov. 28	1.0	"	None.
22	78	" 11 " 13 " 16 " 18	0.5 0.5 0.5 0.5	" " " "	" 28	1.0	"	"
23	140	" 11 " 13 " 16 " 18	0.5 0.5 0.5 0.5	" " " "	" 28	1.0	"	"
19	98	" 11 " 13 " 16 " 18	0.5 0.5 0.5 0.5	" " " "	" 2	0.5	"	"
20	150	" 11 " 13 " 16 " 18	0.5 0.5 0.5 0.5	" " " "	" 2	0.5	"	"

TABLE II.

Rat No.	Weight. gm.	Date sensitized.	Amounts of horse serum.	Method.	Length of time after last in- jection.	Amount of horse serum.	Method.	Symptoms.
13	74	1921 Oct. 24	cc. 0.2	Subcutaneous.	days 38	cc. 0.2	Subdural.	General convulsion; rapid respirations. 4 min., weak. 10 min., still collapsed; weak on left side. 15 min., recovered but paralyzed on left side.
		" 26	0.4	"				
		" 28	0.5	"				
		Nov. 28	1.0	Intravenous.				
14	68	Oct. 24	0.2	Subcutaneous.	38	0.2	"	None.
		" 26	0.4	"				
		" 28	0.5	"				
		Nov. 28	0.5	Intravenous.				
16	75	Oct. 24	0.2	Intraperitoneal.	38	0.5	"	Convulsions; stops breathing. 2 min., recovered. 3 min., gasping. 10 min., dyspnea continued. 1 hr., recovered.
		" 26	0.5	"				
		" 28	0.5	"				
		Nov. 28	1.0	Intravenous.				
17	100	Oct. 24	0.2	Intraperitoneal.	38	0.5	"	Moderate collapse; stops breathing. Respiration started in 1 min. 3 min., crawls; respirations rapid. 6 min., walks about recovering.
		" 26	0.5	"				
		" 28	0.5	"				
		Nov. 28	1.0	Intravenous.				
18	105	Oct. 24	0.2	Intraperitoneal.	38	0.3	"	Immediate collapse. Respiration starts in 1 min. 2 min., deep breathing. 3 min., labored respirations. 7 min., stands; respirations difficult. 18 min., recovered.
		" 26	0.5	"				
		" 28	0.5	"				
		Nov. 28	1.0	Intravenous.				

42	120	1922 Jan. 6	0.2	Intraperitoneal.	27	0.2	Subdural.	Walks about; respirations rapid and slightly difficult. Recovered in 3 min.
Controls. 32	105	" 9	0.5	"				Immediate collapse; convulsions. 2 min., lies on side; hind leg weak. 7 min., better. 15 min., recovery.
		" 11	0.5	"				
		" 13	0.5	"				
		" 16	0.5	"				
		" 18	0.5	"				
33	92					0.2	"	Immediate convulsions; respiration stopped. 2 min., rapid respirations; recovery. 10 min., recovered.
34	105					0.5	"	Immediate shivering; respiration ceases. 2 min., breathing. 3 min., respirations difficult. 6 min., still very sick. 7 min., recovery.
60	115					0.2	"	Immediate collapse; respirations rapid; sneezing; coughing. 6 min., respirations difficult; fluid from nose. 10 min., sneezing; respirations difficult. 30 min., still very sick. 1 hr., recovery.

peritoneally and 1 month later 1 cc. intravenously. The test injections were made from 21 to 44 days after the last sensitizing dose. These experiments are recorded in Tables I and II. In seventeen experiments the injection of 0.5 to 1 cc. of horse serum into the femoral vein of these supposedly sensitized rats (Table I) seemed quite harmless and never produced any symptoms that resembled in the least those obtained with peptone or histamine, much less death. When injections of from 0.2 to 0.5 cc. of horse serum were made subdurally in six rats, immediate and violent symptoms usually occurred, which, however, never proved fatal, and exactly similar symptoms were obtained in the normal control rats (Table II).

From these experiments one must conclude that anaphylaxis to horse serum cannot be obtained in white rats by a second injection of antigen given intravenously or subdurally.

Determination of the State of Sensitization.

The skin of seventeen white rats subjected to repeated injections of horse serum given subcutaneously, intraperitoneally, or intravenously was tested for a specific reaction on the 20th to the 63rd day after the last injection of antigen. The sides of the rat were shaved 2 or 3 days preceding the experiment and an injection of 0.02 cc. of horse serum diluted 1:10 with 0.85 per cent NaCl was made intracutaneously. As controls, similar amounts of rabbit serum diluted 1:10, or of 0.85 per cent NaCl, were given intracutaneously to the inoculated rats and at the same time both horse serum and rabbit serum in similar amounts and dilutions were injected intracutaneously into normal rats. The skin of the rats was observed every 15 minutes for a period of 1 to 2 hours and again at the end of 12, 24, and after 48 hours. In no instance was there any evidence of a skin reaction such as may be obtained in the guinea pig, rabbit, or in man, and in no instance did the test injection produce an effect which differed from that in the controls.

It must be concluded, therefore, that the skin of the white rat subjected to repeated injections of horse serum does not differ from that of the normal rat in its reaction to subsequent intracutaneous injections of horse serum in the amounts utilized.

It is generally assumed that the reactions of the smooth muscle of the sensitized virgin guinea pig as employed by Dale is one of the most delicate tests for sensitization; and for this reason six virgin white rats averaging 50 to 60 gm. were injected intraperitoneally with 0.2, 0.2, 0.5, and 0.5 cc. of horse serum every other day, and after intervals of from 38 to 54 days the uteri were removed, suspended in 250 cc. of oxygenated Locke's solution at 39°C., and tested for their reaction to horse serum. As control antigens, rat serum, rabbit serum, and sheep serum were employed. In five control experiments with the uteri of normal virgin rats, it was found that horse serum in doses of from 1 to 2.5 cc. produced no change in the regular rhythmic uterine contraction but sometimes resulted in a very slight general shortening of the muscle strip which was made evident by an elevation of the line of contractions upon the surface of the drum. The effect of histamine in doses of 1 mg. was to produce immediate relaxation of the muscle strip with great slowing of the contraction, which often became irregular.

The effect of horse serum on the uteri of the inoculated rats in doses of 1 cc. did not differ from that produced by horse serum upon the uteri of normal rats. No unusual contractions, such as are seen in the guinea pig uterus, were observed, and in no instance was the relaxing effect noted with histamine encountered.

It was therefore concluded that the uteri of white rats receiving repeated injections of horse serum did not differ in their reaction to horse serum from those of normal rats.

It seemed possible to conclude, therefore, from these series of experiments that white rats could not be made anaphylactic to horse serum, as tested by several methods. The next step was to determine upon what this refractory condition depended.

This insusceptibility might be explained by at least one of two hypotheses: first, that the tissues of the rat were insusceptible to the poisonous effect which follows the reaction presumably of antibody and antigen in the highly sensitized animal when a second injection of antigen is administered; or, second, that the tissues of the rat were for some reason unable to form an hypothetical anaphylactic antibody or were in some way prevented from accomplishing this.

To answer the first question, experiments were done to determine whether the serum of supposedly immunized rats contained anaphylactin and was capable of transferring passive anaphylaxis to guinea pigs.

Attempts to Sensitize Passively Guinea Pigs with the Serum of White Rats Immunized to Horse Serum.

The serum of fourteen white rats, subjected to repeated intraperitoneal injections of horse serum, was injected within a few hours of bleeding into guinea pigs. Seven guinea pigs received from 0.4 to 0.6 cc. of rat serum intravenously and seven from 0.75 to 1 cc. intraperitoneally. After an interval of 23 to 25 hours these guinea pigs were given 0.5 cc. of horse serum intravenously. No symptoms which could be interpreted as those of anaphylactic shock were observed in any instance. The experiments are summarized in Table III.

These experiments furnish a strong argument, in spite of the resistance of the white rat to peptone and histamine, against the idea that the rat behaves immunologically in the same manner as the guinea pig, rabbit, and dog, but nevertheless escapes the symptoms of anaphylaxis owing to its resistance to the poisonous effects of the shock. It was, consequently, necessary to seek further for an explanation of its insusceptibility to anaphylaxis.

Antigen-Antibody Content of the Serum of White Rats Injected with Horse Serum.

Experiments were therefore instituted to determine whether the second hypothesis was correct and to discover if the same protective mechanism towards sensitization existed in the rat as has been described for the human being. For this purpose a study of the antigen content of the serum of rats injected with horse serum was made. Twenty-one rats were used. Since the size of the rat prohibited the use of a single rat for repeated bleedings, it was necessary to use a single rat for each experiment. The rats, therefore, were killed by bleeding from the heart at various times after a single intravenous injection of approximately 1 cc. of horse serum per 100 gm. of body weight. By employing the precipitin reaction in the manner described

TABLE III.

Guinea pig No.	Weight.	Date.	Rat serum No.	Amount of rat serum.	Method.	Interval.	Amount of horse serum injected intravenously.	Symptoms.
	gm.	1922		cc.		hrs.	cc.	
6	240	Jan. 13	8	1.0	Intraperitoneal.	23	0.5	None.
5	200	" 13	15	0.6	Intravenous.	23	0.5	Shivering; no other symptoms.
10	230	" 19	19	0.5	"	24	0.5	None.
11	240	" 19	20	0.5	"	24	0.5	"
12	180	" 20	35	0.5	"	23	0.5	"
13	190	" 20	36	0.5	"	23	0.5	Shivering; no other symptoms.
14	200	Feb. 1	37	0.4	"	23	0.5	None.
15	225	" 1	38	0.5	"	23	0.5	"
		1919						
19	150	Mar. 8	33	0.75	Intraperitoneal.	24	0.5	Shivering; slight weakness of hind legs. Recovered in 3 min.
20	111½	" 8	34	1.0	"	24	0.5	None.
21	146	" 8	41	1.0	"	24	0.5	"
22	136	" 8	42	1.0	"	24	0.5	Slight shivering; slight weakness; immediate recovery.
23	120	" 8	59	0.2	"	25	0.5	Shivering.
24	223	" 8	60	1.0	"	25	0.5	None.
Controls.								
8	250	Jan. 13					0.5	"
7	230	" 13	Normal.	0.8	Intraperitoneal.	23	0.5	"
9	250	" 20	"	0.5	Intravenous.	48	0.5	"
17	190	Feb. 1	"	0.5	"	23	0.5	Shivering.
25	120	Mar. 8	"	1.0	Intraperitoneal.	24	0.5	None.
						days		
1	220	Jan. 19	Jan. 6. Sensitized to horse serum.			13	0.5	Immediate symptoms; death in 2 min.
2	230	Feb. 1	" 6. "	"	"	25	0.5	" " " "
3	220	Mar. 8	" 6. "	"	"	61	0.5	Death in 2 min.

in previous communications—except that all sera were employed in amounts of 0.25 cc.—it could be shown that antigen (horse serum) when injected intravenously did not persist in the circulation but disappeared in from 12 to 14 days, a period which is approximately that required by the rabbit to dispense with a similar antigen.

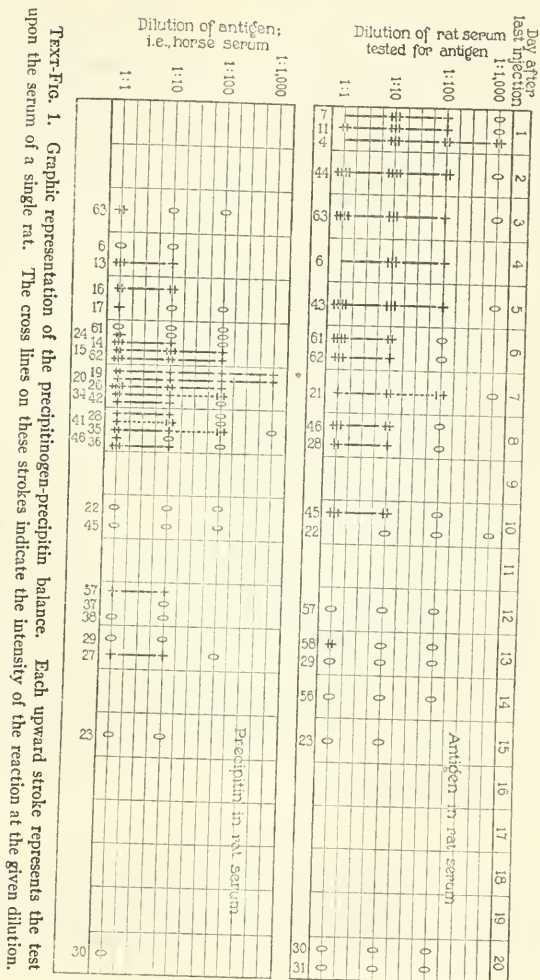
It was thought that the kidney of the rat might be permeable to the horse serum and thus allow of its escape from the body, but precipitin reactions upon the urine of white rats injected with horse serum failed to demonstrate antigen in the urine from the time of injection to the time of the disappearance of the horse serum from the circulation.

It was necessary, therefore, to search for some other explanation. Although it seemed *a priori* improbable that the white rat which was refractory to sensitization would form precipitins, still tests were made for specific precipitins in the serum of twenty-eight white rats receiving single and repeated injections of horse serum, and it was found that these animals were capable of forming such precipitins in fairly high concentration. Precipitins for horse serum could be demonstrated in the serum of twenty of the twenty-eight rats, appearing first on the 3rd to the 4th day after the last of a series of inoculations and increasing in intensity up to the 6th or 8th day, when they were present with great regularity. After this time, there was a rapid decline in positive reactions, for the sera from only two of nine rats drawn after the 10th day showed precipitins. It was also found that it was during the high concentration of precipitins in the serum that the antigen disappeared from the circulation. A graphic presentation of the precipitinogen-precipitin balance is shown in Text-fig. 1.

The mechanism of precipitin formation and the relationship between precipitin and precipitinogen (horse serum) proved, therefore, to be much the same as it is in the rabbit.

Thus, though sensitization to horse serum did not occur, the cells of the white rat had apparently an avidity for horse serum which was as great as, or even greater than that of the rabbit.

Though there has been considerable discussion as to the identity of the anaphylactic antibody and precipitin, the work of Doerr and Russ (12) in rabbits and the subsequent observations of Weil (13) have lent such strong support to the idea that these two bodies are one and the same, that most investigators (Doerr (5), Zinsser (14), Wells (15))



have accepted this explanation as the correct one. Doerr in particular, after a complete review of the literature, has come to this conclusion. It has, however, been generally known that the ease with which an animal may be sensitized to foreign proteins and the readiness with which it forms specific precipitins are not always parallel. The guinea pig, for instance, is highly subject to sensitization but is an extremely poor precipitin producer, whereas the rabbit, which is much more difficult to sensitize, produces precipitins in large amounts.

The present experiments seem to demonstrate that in white rats precipitins and anaphylactin are not identical and that good precipitin formation can occur without anaphylactic sensitization and without the appearance of anaphylactic antibody in the serum of the rat.

The differences which several species of animals show in the ease with which they may be sensitized to foreign proteins and the readiness with which they may be subjected to shock by a second injection of the same antigen have already been alluded to, and it now seems from these experiments that the rat stands at one end of the scale, as a completely refractory animal, and probably the guinea pig at the other, as representing the most highly susceptible animal. This refractory condition of the rat does not seem to be due to a resistance of specialized tissues to the toxic effect of anaphylactic shock, or to an inability of the tissues to absorb antigen, for on the one hand, the animal may be killed by histamine or peptone in large doses, and on the other, the tissues are capable of producing precipitins in considerable concentration but not anaphylactin. The explanation seems rather to be found in the fact that the preliminary injections of antigen, though capable of calling forth precipitins, fail to prepare the cells of the body in the manner necessary to make them vulnerable either by an antibody-antigen reaction or by some other mechanism to the second injection of antigen.

SUMMARY.

1. Attempts to produce anaphylactic shock in white rats by second intravenous or subdural injections of horse serum have failed.
2. It was impossible to demonstrate either by skin reactions or by the uterine reaction that white rats can be sensitized to horse serum.

3. It was not possible to sensitize guinea pigs passively with the serum of white rats presumably immunized to horse serum.

4. In spite of the fact that the white rat could not be made anaphylactic to horse serum, the tissues of the animal reacted with the horse serum to form precipitins in fair concentration and the antigen disappeared from the circulation soon after the precipitins reached their greatest concentration in the blood.

5. These experiments would indicate that in the white rat anaphylaxis and precipitin formation are independent and represent different types of immunological processes.

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LEUCOCYTIC SECRETIONS.

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I.

INTRODUCTION.

Leucocytes may be regarded as mobile unicellular glandular bodies which set free their secretions in the humors of the organism. But little is known of the nature and functions of the substances they secrete. In the following experiments, leucocytes were cultivated *in vitro* and a study was made of certain modifications of the medium caused by their secretions.

II.

Technique.

The leucocytes were obtained from hen blood and cultivated in Gabritschewski dishes. The serum was extracted from the culture medium and the variations of its inhibiting action on homologous fibroblasts, and of its lytic action on foreign erythrocytes, were measured.

1. *Preparation of the Leucocytes.*—The blood was taken from young adult chickens which had fasted for 24 hours. The animals were bled from the carotid artery or from one of the veins of the wing, through an oiled cannula or syringe. The blood was received in cold paraffin-coated tubes and centrifuged in ice for 10 minutes. After the plasma had been removed, a few drops of Ringer solution containing a small amount of embryo juice were placed at the surface of the corpuscles and the tubes were allowed to stand at room temperature for about 15 minutes. Then the film of leucocytes, which covered the erythrocytes, became solid, and could be removed to a watch-glass filled with Ringer solution. The film was washed almost free of erythrocytes and cut in small fragments of about equal size.

2. *Preparation of the Medium.*—The medium consisted of chicken plasma and embryo tissue juice. Its composition was generally as follows:

Chicken plasma.....	5.00 volumes.
Embryonic tissue juice.....	0.25 volume.
Tyrode solution.....	2.75 volumes.
Distilled water.....	2.00 volumes.

The medium was rendered hypotonic on account of the evaporation which takes place in the Gabritschewski dishes. In some cultures, casein was added to the medium. 1 gm. of casein was dissolved in a diluted solution of sodium hydroxide, and dialyzed against tap water for 3 days. Then it was again brought into solution by the addition of a little alkali, and rendered isotonic by a proper amount of NaCl. The pH of the solution was about 7.8. The amount of the solution used in the medium varied from 0.1 to 1 volume of a 1 per cent casein solution; that is, the concentration of casein was from 0.1 to 1 per 1,000.

3. *Preparation of the Cultures.*—The cultures were made on Gabritschewski dishes, the covers of which had been replaced by mica plates. The fragments of leucocytic films were suspended in 5 volumes of Tyrode solution, distilled water, and embryo juice in the concentrations previously described. The same number of fragments was used for each culture. Then 5 volumes of plasma were spread on the mica plate, with the necessary precautions to prevent bacterial contamination from the air. Afterwards, the leucocytic suspension was dropped from a pipette into the plasma, and thoroughly mixed with it. Care was taken to distribute the fragments of leucocyte film evenly. Their number was about thirty to each dish, in order that the colonies might grow freely around each fragment, without being in contact with the edges of the neighboring colonies before 48 hours. Controls were prepared consisting of the medium without leucocytes. Both cultures and their controls were incubated for 48 hours at 38°C. Other cultures and controls were placed in the refrigerator for the same time.

4. *Preparation of the Serum from the Cultures.*—After 48 hours, the cultures were examined. Around each fragment, a large colony of leucocytes had grown. The edges of the colonies were generally in close contact. Microscopic examination showed whether the cells

were living and in active condition, and whether bacterial contamination had occurred. Then the boxes were opened and the coagulum was sliced with fine scissors. The fluid and the fragments of coagulum were centrifuged at high speed for 10 minutes. The supernatant fluid was removed and its H ion concentration ascertained by the technique described by Felton.¹ The serum of the cultures of leucocytes always became slightly acid after 48 hours in the refrigerator. When the colonies had grown actively in the incubator for 48 hours, the acidity of the medium was much more marked. The pH of the serum from the cultures was brought to about that of normal serum by adding a small amount of sodium hydroxide.

5. *Action of Serum on Chicken Fibroblasts and on Foreign Erythrocytes.*—The sera from cultures with and without leucocytes, incubated or kept in the refrigerator, were compared from the standpoint of both their hemolytic effect on sheep or rabbit erythrocytes and their inhibiting power on chicken fibroblasts. The hemolytic action is expressed in the tables by the amount of serum required for a given degree of hemolysis. The action on chicken fibroblasts was ascertained by the rate of growth of a 10 year old strain of fibroblasts in a medium composed of 2.5 volumes of chicken plasma, 5 volumes of serum from leucocyte cultures, 2.2 volumes of Tyrode solution, and 0.3 volume of embryonic juice. In the tables, the rate of growth is indicated by the relative increase of the fragment of fibroblasts in 48 hours. The quotient of the average rate of growth of the experiment by the average rate of growth of the control expresses the action of serum on homologous fibroblasts.

III.

RESULTS.

The experiments consisted in measuring the inhibiting effect on chicken fibroblasts and the hemolytic power on sheep erythrocytes of the serum from media without leucocytes and of the serum from similar media with leucocytes, after the colonies had been allowed to grow for 48 hours. Other experiments were made in order to study the action of temperature on serum and cells. A culture medium without

¹ Felton, L. D., *J. Biol. Chem.*, 1921, xlii, 299.

TABLE I.

Action of Serum from Cultures of Chicken Leucocytes on Homologous Fibroblasts and Sheep Erythrocytes.

Experiment No.	Culture No.	Rate of growth in serum from cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
		Without leucocytes (C).	With leucocytes (E).		Amount of serum.	Amount of hemolysis by serum from cultures.	
						Without leucocytes.	With leucocytes.
1	1410	1.50	3.00	2.00	per cent 0	per cent 0	per cent 0
2	24858-1	2.65	3.00	1.13	0	0	0
		2.85	3.84				
		2.74	2.56				
3	24858-2	2.19	4.60	1.51	0	0	0
		3.27	4.10				
		2.82	3.44				
4	25171	1.94	3.05	1.60	0	0	0
		2.36	3.65				
		1.86	3.17				
5	25213	2.53	3.97	1.57	0	0	0
		2.94	4.46				
		2.66	4.28				
		3.40	5.43				
6	25391	2.77	3.90	1.45	0	0	0
		2.40	3.35				
		2.02	3.64				
		3.28	4.00				
7	28616	2.59	4.57	1.71	0	0	0
		3.00	4.94				
		2.57	4.16				
8	28801	3.07	5.41	1.56	0	0	0
		3.19	4.37				
9	28684	1.62	3.40	2.00	70	0	40
		2.13	4.09		50	0	20
					30	0	5
10	28641	2.66	4.57	1.37	70	0	45
		4.13	4.29		50	0	25
					30	0	5

TABLE I—*Concluded.*

Experiment No.	Culture No.	Rate of growth in serum from cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
		Without leucocytes (C).	With leucocytes (E).		Amount of serum.	Amount of hemolysis by serum from cultures.	
						Without leucocytes.	With leucocytes.
11	28770	6.18	7.00	1.70	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
		3.38	7.68		70	0	0
12	29263	4.41	9.07	2.18	80	7.00	10
		2.57	6.30		40	5.00	10
		2.60	5.29		20	2.50	4
13	28801	3.07	5.41	1.56	70	0	0
		3.19	4.37				
14	28886	2.38	1.70	1.10	70	0	40
		3.10	4.66		50	0	30
					30	0	8
15	28926	0	0		80	5.00	40
				60	2.50	20	
				20	2.50	5	
				10	2.50	2.50	
16	29019	0	0		80	10.00	20
				60	8.00	15	
				40	5.00	8	
				20	2.50	3	
17	29162	1.21	6.77	3.33	80	0	0
		3.20	5.15				
		2.26	6.38				
18	29400	0	0		80	60.00	75
				60	50.00	55	
				40	30.00	30	
19	29059	1.87	4.14	1.98	80	0	0
		3.86	6.70				
		3.38	6.80				

leucocytes kept in the refrigerator for 48 hours was compared with a similar medium incubated for the same length of time. The sera from culture media with and without leucocytes, kept in the refrigerator instead of being incubated, were also compared.

1. *Serum from Media Incubated for 48 Hours, with and without Leucocytes.*—In every case, the colonies grew actively and were still living after 48 hours. In seventeen experiments (Table I), the serum from the media with leucocytes was more favorable to the activity of fibroblasts. The quotient of the rate of growth in serum from leucocyte cultures divided by the rate of growth in serum from media without leucocytes averaged 1.73.

TABLE II.

Action of Serum from Culture Media, Incubated and Refrigerated, on Homologous Fibroblasts and Foreign Erythrocytes.

Experiment No.	Culture No.	Rate of growth in serum from culture media.		Ratio, $\frac{E}{C}$.	Hemolytic action on foreign erythrocytes.		
		Incubated (C).	Refrigerated (E).		Amount of serum.	Amount of hemolysis by serum from culture media.	
						Incubated.	Refrigerated.
1*	29094	1.25	6.53	4.49	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
		1.68	6.33		80	8	80
					20	5	50
					10	0	25
2†	29339	1.04	2.38	3.20	70	15	80
		0.87	3.73		50	8	80
		1.34	4.10		30	3	70
					80	55	75
3‡	29436	0	0		40	40	70
					10	10	20

* In Experiment 1, sheep erythrocytes were used.

† In Experiments 2 and 3, rabbit erythrocytes were used.

The hemolytic power of the serum on sheep or rabbit erythrocytes was measured in eleven experiments. In four cases (Table I), both sera were inactive. In seven cases (Table I), the sera from cultures without leucocytes were inactive, while the sera from cultures with leucocytes were hemolytic for sheep or rabbit corpuscles. After the serum had been heated at 56°C., its hemolytic action disappeared completely.

(a) *Serum from Culture Media without Leucocytes, Incubated and Refrigerated.*—The effect of heat at 38°C. for 48 hours on media without leucocytes was examined (Table II). The natural hemolytic action of the serum on sheep or rabbit corpuscles decreased after the medium had been incubated, while it remained normal when the medium was kept in the refrigerator. At the same time, the serum from the incubated medium became much less favorable to the growth of chicken fibroblasts (Table II).

TABLE III.

Action of Serum from Cultures of Chicken Leucocytes, Refrigerated, on Homologous Fibroblasts and Sheep Erythrocytes.

Experiment No.	Culture No.	Rate of growth in serum from cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
		Without leucocytes (C).	With leucocytes (E).		Amount of serum.	Amount of hemolysis by serum from cultures.	
						Without leucocytes.	With leucocytes.
					<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	28684	1.33	2.14	1.31	70	40	60
		1.92	2.03		50	20	20
		1.91	2.50		30	5	10
2	28641	5.20	3.83	0.73	70	70	80
					30	10	30
3	28770	4.83	4.82	0.99	50	100	100
		7.93	7.85		30	80	35
4	29263	4.06	4.55	1.91	60	35	35
		2.59	5.16		40	8	20
		2.85	7.47		20	2.5	10
					10	2.5	5

(b) *Serum from Culture Media with and without Leucocytes, Refrigerated for 48 Hours.*—In every experiment (Table III), the hemolytic power of the serum from media without leucocytes remained normal, while that from media with leucocytes was slightly increased. At the same time, there was no marked difference in the average rates of growth of fibroblasts in both sera.

2. *Serum from Media with and without Leucocytes, to Which a Foreign Protein Was Added.*—When 1 per 1,000 casein was added to a culture medium containing no leucocytes, the serum became more inhibiting

for the growth of fibroblasts. It was less toxic when the medium contained only 0.1 per 1,000 casein. A comparison of both sera from cultures of leucocytes with and without 1 per 1,000 casein showed that the presence of casein increased the amount of leucocytic secretion. The juice of the cultures containing casein was always a better medium for homologous fibroblasts than that of the cultures without casein, in spite of the toxic action of the casein (Table IV).

TABLE IV.

Action of Serum from Cultures of Chicken Leucocytes, without and with Casein, on Homologous Fibroblasts and Sheep Erythrocytes.

Experiment No.	Culture No.	Casein concentration in medium.	Rate of growth in serum from leucocytic cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
			Without casein (C).	With casein (E).		Amount of serum.	Amount of hemolysis by serum from leucocytic cultures.	
							Without casein.	With casein.
						<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	28801	1 per 1,000	4.49	8.80	1.71	70	0	0
			4.16	7.70				
			7.90	10.50				
2	28886	1 " 1,000	4.81	5.35	1.34	70	40	40
			2.75	4.23		50	30	30
						30	8	8
3	1410	Dog serum.	1.00	2.50	2.50	0	0	0
4	28988	0.1 per 1,000	2.05	2.10	1.03	80	0	0
			1.54	1.32				
			1.81	2.21				
5	29059	0.1 " 1,000	6.71	6.70	1.17	80	0	0
			5.31	7.25				

The quotient of the rates of growth of fibroblasts in serum from casein media with and without leucocytes was higher than the quotient of the rates of growth of similar cultures containing no casein (Table V). There was little or no difference in the action of the sera when the concentration of the casein in the culture media was 0.1 per 1,000 (Table V).

TABLE V.

Action of Serum from Cultures Containing Casein, without and with Chicken Leucocytes, on Homologous Fibroblasts and Sheep Erythrocytes.

Experiment No.	Culture No.	Casein concentration in medium.	Rate of growth in serum from cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
						Amount of serum.	Amount of hemolysis by serum from cultures.	
			Without leucocytes (C).	With leucocytes (E).			Without leucocytes.	With leucocytes.
						per cent	per cent	per cent
1	28801	1 per 1,000	1.00	4.04	4.40	70	0	0
			0.95	4.84				
			1.13	4.63				
2	28886	1 " 1,000	1.00	1.94	2.03	0	0	0
			1.00	2.00				
			1.14	2.10				
3	28926	0.5 per 1,000	0	0		80	5	50
						60	3.5	30
						40	2.5	15
4	29019	0.5 " 1,000	0	0		80	10	25
						60	8	20
						20	2.5	5
5	29162	0.5 " 1,000	2.88	10.00	4.39	80	0	0
			1.25	5.76				
			1.69	8.66				
6	29400	0.5 " 1,000	0	0		80	60	75
						60	50	65
						40	30	40
7	29059	0.1 " 1,000	6.80	12.77	2.06	80	0	0
			4.81	10.35				
			2.61	5.68				
8	29435	0.5 " 1,000	0.65	2.93	4.34	0	0	0
			1.19	4.90				

No increase of the lytic action on foreign erythrocytes of the serum from culture media containing casein was observed.

IV.

DISCUSSION, SUMMARY, AND CONCLUSIONS.

In the present condition of the technique of cultivation of tissues, the only possible way of studying leucocytic secretions was to grow colonies of leucocytes in a medium of known properties and to examine the modifications of these properties under the influence of the living cells. The method was far from perfect, because the secretions were mixed with serum and accumulated for 48 hours in a medium where they probably underwent partial destruction. But an approximate idea of certain of the qualities of the secretions, although not of their quantity, could be derived from the experiments. In the fluids extracted from the cultures, we attempted to detect the presence of the leucocytic secretions through their physiological effects on homologous and foreign cells. Two kinds of substances were sought, those which act on homologous cells, and those which destroy foreign erythrocytes.

The secretion by leucocytes of substances necessary to the nutrition of other cells was considered as probable long ago. Renaut² thought that the main function of the white blood corpuscles was to bring to the fixed cells of the tissues the food material which they need. While the existence of physiological relations between leucocytes and tissue cells could be considered as almost certain, their nature had remained practically unknown. It was probable, however, that the substances secreted by leucocytes were analogous to the growth-activating and unstable substances which are found in embryonic tissues, leucocytes, and certain adult tissues.³ When connective tissue was aseptically inflamed, or when an aseptic peritoneal exudate contained many leucocytes, aqueous extracts of both connective tissue and peritoneal exudate were found to have acquired the power of stimulating cell proliferation.⁴ These experiments showed that leucocytes could bring to the tissues some activating substances. But it remained to be ascertained whether leucocytes, while they are alive, could secrete similar substances either spontaneously or under the stimulus of a foreign factor.

✓ ² Renaut, J., *Traité d'histologie pratique*, Paris, 1893, i, pt. 2, 968.

✓ ³ Carrel, A., *J. Exp. Med.*, 1913, xvii, 14; xviii, 287.

✓ ⁴ Carrel, A., *J. Exp. Med.*, 1922, xxxvi, 385.

Leucocytes are supposed to be, as is well known, the origin of the substances which protect the organism against infection.⁵ Although the problem of the origin of alexin and antibodies has been investigated by many experimenters, it is not yet completely solved. It was of interest, therefore, to ascertain whether leucocytic secretions could increase the natural hemolytic effect of hen serum on sheep or rabbit erythrocytes, and whether these secretions would become more active under the influence of a foreign protein. The substances which destroy foreign cells are not necessarily different from those which act on homologous cells. The word substance is used for simplicity of description and may be taken as meaning only a given property of an unknown substrate.

A comparison was made of certain properties of sera extracted after 48 hours incubation from media containing leucocytes and from media containing no leucocytes. The serum from the leucocytic cultures was always found to be more favorable to the growth of homologous fibroblasts than the serum from the culture media incubated without leucocytes. The natural hemolytic power of the serum on sheep erythrocytes was found to be increased in about 50 per cent of the experiments.

In other experiments, we found that when two culture media free of cells were placed, one in an incubator at $+38^{\circ}\text{C}$. and the other in a refrigerator at $+5^{\circ}\text{C}$. for 48 hours, the serum from the incubated medium partly lost its hemolytic action on sheep or rabbit erythrocytes, while that from the refrigerated medium remained normal; at the same time, the inhibiting action of the incubated medium on homologous fibroblasts had increased very much. This effect of incubation indicates that certain unstable constituents of serum are destroyed by heat. Then the changes found in the properties of the serum from cultures of leucocytes are due to the fraction of the activating substances which has not been destroyed by incubation at 38°C . A quantitative study of the secretions is, therefore, impossible with the present technique, which can furnish only qualitative indications about the substances set free by the leucocytes.

⁵ Metchnikoff, E., *Immunity in infective diseases*, translated by Binnie, F. G., Cambridge, 1905.

We have ascertained also whether a medium containing leucocytes and kept in the refrigerator undergoes any change under the influence of the cells while they are in a condition of latent life. Gabritschewski dishes with and without leucocytes were placed in a refrigerator at a temperature of about $+5^{\circ}\text{C}$. After 48 hours, the hemolytic power on sheep erythrocytes of the serum from the leucocytic cultures had increased slightly and its inhibiting action on the growth of homologous fibroblasts had decreased. Then certain substances favorable to the growth of homologous cells and toxic for heterologous cells were diffused by the leucocytes into their medium. But the action of these substances was weaker than in the case of the cultures kept in the incubator. This experiment showed that leucocytes under certain conditions diffuse alexin or natural hemolysins which originate from them at the same time as the substances which activate homologous cells. In other experiments, although leucocytes were frozen at -10°C ., treated with distilled water, or extracted with saline solution, they did not yield any hemolysin.

To summarize: Leucocytes, cultivated in plasma, always secreted substances which increased the rate of growth of homologous cells. Less frequently, they set free substances which hemolyzed foreign erythrocytes.

The growth-promoting substances are analogous to those contained in embryonic tissues, and probably represent some of the foodstuffs brought to fixed tissue cells by leucocytes. They may possess the function of rejuvenating cells which have ceased to multiply when the cicatrization of a wound or the repair of a fracture requires a resumption of tissue activity.⁴ According to this hypothesis, the leucocytes brought to the surface of a wound by the process of inflammation would not only oppose bacterial invasion, but also bring to the tissues the material necessary to cell multiplication. It seems that in some cases regeneration is started by substances brought to the tissues by other cells. Loeb thinks that in *Tubularia*, when endodermic cells gather at the end where a new polyp is about to be formed, the substances given off by these cells are responsible for polyp formation.⁶ There may be

⁶ Loeb, J., *The organism as a whole from a physicochemical viewpoint*, New York and London, 1916, 170.

an analogy between this phenomenon and the secretion by leucocytes of growth-activating substances at the surface of a wound.

If we assume that leucocytes *in vivo* set free their secretions in the blood stream, certain variations of the growth-inhibiting action of normal serum can be better understood. The rate of proliferation of homologous fibroblasts is much slower in the serum of an old chicken than in that of a young one.⁷ When the serum is heated at 56° and 70°C. for $\frac{1}{2}$ hour, it becomes still more inhibiting.⁸ A substance favorable to cell activity has disappeared. It is therefore permissible to suppose that the growth-inhibiting power of serum and its variations are due to the antagonistic action of two substances, one growth-promoting and thermolabile, and the other growth-inhibiting and thermostable, the activating substance being always weaker in its effect than the inhibiting one. We know that activating substances can be extracted from embryonic tissue, from muscle and gland tissues, and from leucocytes of the adult animal, and that they are thermolabile and very unstable. Leucocytic secretions seem to have some of the properties of leucocytic extracts. It is probable that the activating substances which disappear from the heated serum are secreted by leucocytes and other cells. An increase of these secretions, then, would diminish the inhibiting action of serum on homologous fibroblasts. On the contrary, a decrease of the secretions in the serum would increase its inhibiting effect on homologous cells. The strong inhibiting action of serum in old age would be due partly to a reduction in the amount and activity of the substances secreted by leucocytes and tissue cells in the humors of the organism.

Leucocytes also secreted *in vitro* substances which were toxic for foreign cells. Although the results were not constant, the serum appeared to become slightly more hemolytic for sheep or rabbit erythrocytes, under the influence of the leucocytes. The hemolysis of rabbit corpuscles by hen serum is due, according to Hyde,⁹ to a complex sensitizer alexin, and not merely to alexin, as Bordet thought.¹⁰

⁷ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.

⁸ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 647.

⁹ Hyde, R. R., *Am. J. Hyg.*, 1921, i, 358.

¹⁰ Bordet, J., quoted by Hyde,⁹ p. 358.

When a foreign protein was added to the culture medium, the leucocytic secretions increased, as was shown by the action on homologous fibroblasts of sera taken from cultures of leucocytes with and without casein. The presence in the medium of the cultures of leucocytes of only 0.1 per 1,000 casein did not markedly modify the action of their serum on the proliferation of fibroblasts. When the concentration of casein in the leucocyte cultures reached 1 per 1,000, the growth of chicken fibroblasts in the serum extracted from the Gabritschewski dishes became more rapid. But there was no parallel increase of the hemolytic action of the serum upon sheep erythrocytes.

We found that chicken serum containing 0.1 per 1,000 casein was barely toxic for homologous fibroblasts, while it became markedly inhibiting when the casein concentration reached 1 per 1,000. Probably, there is a relation between the toxicity of the medium, the increase of leucocytic secretions, and the time of the increase. The change brought about by casein in the equilibrium of the system composed of the cells and their medium determines the secretion by the leucocytes of substances which increase the activity of homologous cells and oppose the inhibiting effect of the foreign proteins. This reaction of the leucocytes is immediate, and may represent the first defense of the organism against a factor which disturbs its equilibrium. Possibly it differs from the specific cell reaction which leads to the production of antibodies. It is known that antibodies develop more slowly. Hemolysins were detected in cultures of bone marrow 4 days after the addition of antigen.¹¹ The immunization of fibroblasts against foreign proteins has been shown by Fischer¹² to begin after 4 days. If leucocytes behave in the organism as they do *in vitro*, we may assume that before the appearance of antibodies, they respond to the presence of an antigen by setting free growth-promoting substances and possibly alexin. This immediate reaction of the leucocytes against a disturbing factor, and the resulting production of substances which increase the activity of homologous cells, might be partly responsible for the results observed in the treatment of certain diseases by the injection of foreign proteins.

¹¹ Carrel, A., and Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 287.

¹² Fischer, A., *J. Exp. Med.*, 1922, xxxvi, 535.

It may be concluded that, under the conditions of the experiments:

1. The serum obtained from cultures of leucocytes is less inhibiting for homologous fibroblasts than the serum from media without leucocytes. In some experiments, its hemolytic action on sheep or rabbit erythrocytes is also increased.

2. The addition of casein to leucocytic cultures brings about a decrease in the inhibiting effect of the serum on homologous fibroblasts.

3. The increase in the activity of homologous fibroblasts in serum obtained from leucocytic cultures is probably due to growth-promoting substances secreted by the leucocytes. The presence of a foreign protein under certain conditions determines a more abundant leucocytic secretion.

THE LOSS OF CIRCULATING ERYTHROCYTES IN CERTAIN TYPES OF EXPERIMENTAL PNEUMONIA.

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During the course of a prolonged investigation of experimental pneumonia, a red blood cell count was made as a routine procedure. Our first thought was that there would be a compensatory rise in the number of erythrocytes to counteract the effect of interference with blood oxidative processes in the lung as a result of the pneumonic lesions. We found otherwise.

In the work on pneumonia some 60 dogs were employed. Of these, 36 were studied for changes taking place in the blood.

1. Inoculated with *B. bronchisepticus*, 14 animals (Nos. 1 to 14).
8 satisfactory: 2 killed, 3 died, 3 recovered.
6 unsatisfactory: 1 died too soon, 5 ineffective inoculations.
2. Inoculated with *Streptococcus hemolyticus*, 3 animals (Nos. 16, 17, and 18).
1 satisfactory, recovered.
2 unsatisfactory, ineffective inoculations.
3. Inoculated with *Streptococcus viridans*, 2 animals (Nos. 20 and 24).
1 satisfactory, recovered.
1 unsatisfactory, ineffective inoculation.
4. Inoculated with *Pneumococcus* Type I, 1 animal (No. 21).
1 unsatisfactory, ineffective inoculation.
5. Inoculated with *B. influenza*, 1 animal (No. 22).
1 satisfactory, recovered.
6. Inoculated with *B. mucosus capsulatus*, 39 animals (Nos. 15, 19, 23, and 25 to 60).
25 satisfactory: 1 killed, 16 died, 8 recovered.
14 unsatisfactory: 7 died too soon, 2 inadequately observed, 5 ineffective inoculations.

It is to be noted that 46 of the dogs were successfully inoculated and showed clinical symptoms of pneumonia. Of this number, eight died within 18 hours of inoculation, so that it was impossible to obtain observations upon them.

Inoculations were made by tracheal insufflation (1). Fifteen to twenty cultures of a 24 hour growth of the organisms on Löffler's slants were suspended in 10 cc. of Ringer's solution. The animal was anesthetized and a small rubber tube was passed down the trachea through the mouth to the tracheal bifurcation. The suspended organisms were injected through this tube by syringe, and were immediately followed by 10 cc. of Ringer's solution, and then by 10 cc. of air. The purpose of this was to blow the trachea clean, and to spread the organisms as far as possible into the bronchial tree. The dog was kept under ether for 5 minutes longer to prevent the coughing up of any of the organisms.

In the successfully inoculated and observed animals, lung lesions were followed by fluoroscope and x-ray examination; 20 cc. of blood were drawn daily until death or recovery, for the different blood determinations, and lung ventilation was daily recorded by means of a body plethysmograph. A report on these observations will be made at some later time. The blood was drawn from the femoral artery into an oxalated syringe through the intact skin, with suitable aseptic precautions and means to prevent hemorrhage. In this manner circulating blood was obtained for the counts, obviating any chance of securing blood diluted with serum as in the cases in which blood is drawn from ear punctures. The dilution resulting from washing the syringe with sterile saturated potassium oxalate solution before drawing the blood may be considered negligible.

Erythrocyte counts in duplicate, with the American standard hemocytometers with Levy counting chambers certified by the Bureau of Standards, were made on thirty of the thirty-six satisfactory dogs. In all these animals a marked decrease in red cells was noted as the disease progressed. If the animal recovered, the tendency to return to a normal count was not pronounced or in proportion to the speed of recovery. A typical animal, for instance, inoculated with *Bacillus bronchisepticus* (No. 6) had a normal erythrocyte count of 6.6 million per c.mm. 2 days after inoculation, with both

lungs diffusely shaded, the count was 5.2 million. 2 days later, with the right lung densely shaded, and the dog very sick, the count had fallen to 4.4 million, and 3 days later, just before the animal died, the count was 3.6 million. In a dog which recovered from a *Bacillus bronchisepticus* infection (No. 9), the normal erythrocyte count was 6.8 million; 3 days after inoculation, with marked distemper and the left lung diffusely shaded, the count was 5.1 million; the next day, with both lungs cloudy, the count was 4.5 million; on the following day, however, with the lungs clearing and the animal's physical condition markedly improved, the count was 4.2 million; and 2 days later, with the lungs clear, the count had risen only to 5.2 million. The same general effect was noted in the animals successfully inoculated with streptococcus and influenza organisms. Typical erythrocytic effects after inoculation with *Bacillus mucosus capsulatus* are shown in Table I.

The uniformity of the results raised the question as to whether there was an actual loss of circulating erythrocytes, or whether the blood was merely diluting. Accordingly, blood volume determinations by means of the method of Keith, Rowntree, and Geraghty (2), hemoglobin determinations by Palmer's method (3), and hematocrit determinations were made on a small number of the dogs successfully inoculated with *Bacillus mucosus capsulatus*. The results are shown in Table I. Blood volume, within the limits of error of the method, remained constant; the hemoglobin fell approximately in proportion to the fall in the number of red cells, and there was a marked increase in plasma with a corresponding fall in the mass of formed elements. These considerations indicate an actual loss of erythrocytes in the effective circulation, without a compensatory regeneration.

Histological search through the tissues of the various organs after death revealed the red cells in different stages of degeneration in large quantities in the capillaries and tissue spaces of the lungs, liver, spleen, and intestinal walls. The capillaries in all these organs were fairly gorged with red cells, and even in the capillary network of the glomeruli of the kidney there were more red cells than in normal animals. The tissue spaces did not contain erythrocytes except in the lungs and spleen. The latter held a great mass of red cells in degeneration.

The literature on the subject of the variations in the number of erythrocytes in pneumonia is scanty. Cabot (4) notes clinically that there may be a slight anemia following the crisis in pneumonia, and cites Grawitz as stating that the post-critical relaxation of peripheral vessels causes a dilution of the blood with an apparent loss of red cells. Rosenow (5) cites Boeckman as saying that the number of erythrocytes is proportional to the degree of temperature in pneumonia, and himself declares that there is scarcely any infection in which the production of

TABLE I.

Effect of Inoculation with B. mucosus capsulatus on Erythrocytes in Dogs.

Dog No.	Date.	Weight.	Erythro- cytes per c.mm.	Hemo- globin.	Blood volume.	Plasma.	Corpuscles.
	1921	kg.	million	per cent	cc.	per cent	per cent
49	Aug. 4	9.1	7.2	125		54	46
Died.	" 5	9.0	6.1	105		58	42
51	" 15	6.6	7.3	115	590	54	46
Recovered.	" 16	6.4	6.0	101	610	59	41
52	" 16	9.6	7.5	127	1,090	44	56
	" 17	9.5	5.6	104	1,060	51	49
	" 18	9.4	5.5	101	1,030	52	48
Recovered.	" 19	9.5	6.3	110	960	52	48
57	Nov. 3	9.2	7.2	102		54	46
	" 5	9.1	7.0	101	980	54	46
Died.	" 7	8.0	4.2	53	920	69	31
59	" 17	5.8	6.3	105	600	59	41
	" 21	5.6	5.3	90	610	63	37
Died.	" 22	5.1	4.2	76		65	35
60	" 23	8.0	7.2	97		42	58
Died.	" 25	6.8	5.3	80		53	47

The figures for the 1st day in each case are normal, before inoculation.

anemia is so rapid as in pneumonia. Burnett (6), however, in discussing croupous pneumonia in horses, notes that the number of erythrocytes does not greatly change, except sometimes to increase slightly because of the concentration of the blood due to loss of fluid.

We observed that three of the animals in which pneumonia had been induced, although they were in fair condition while lying in

their own chosen curled up position, quickly became dyspneic and cyanotic when placed on their backs.

Erythrocyte counts, blood volume, hemoglobin, and hematocrit determinations were made upon one control animal. This dog lost approximately the same amount of blood as the others, and was subjected to the same manipulative, fluoroscopic, and x-ray procedures, with the exception that it was not inoculated with any organism. The results are shown in Table II. It may be seen that such variations as occurred in the determinations were insignificant.

TABLE II.

Control Dog.

Date.	Weight.	Erythrocytes per c.mm.	Hemoglobin.	Blood volume.	Plasma.	Corpuscles.
1921	kg.	million	per cent	cc.	per cent	per cent
Oct. 7	7.2	6.2	98	800	51	49
" 10	7.3	6.4	100	790	52	48
" 14	7.2	6.1	97	740	54	46

SUMMARY.

Erythrocyte counts, blood volume, hemoglobin, and hematocrit determinations on a series of dogs suffering from experimental pneumonia induced by the tracheal insufflation of *Bacillus bronchisepticus* and *Bacillus mucosus capsulatus* indicate that in this condition there is an actual loss of circulating erythrocytes, without compensatory regeneration.

Postmortem examination of the organs of these animals revealed red cells in various stages of degeneration in the capillaries and tissue spaces of the lungs, liver, spleen, and intestinal walls.

We wish to thank Professor W. J. Meek and Professor P. F. Clark for encouragement in this work.

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THE SUPRAVITAL STAINING OF VACCINE BODIES.

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PLATES 58 AND 59.

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INTRODUCTION.

To determine the nature of the conspicuous cell granulations found in the lesions of vaccinia is a problem in cytology of unusual interest which has continued to hold the attention of investigators.¹ They seem to represent the visible expression of a fundamental cellular reaction. From the historical point of view three lines of thought may be recognized in the development of our present knowledge of the subject, which, in its broader aspects, cannot well be considered apart from other diseases of unknown etiology.

1. On their discovery, the granulations were first named after their observers, Guarnieri's bodies in vaccinia and variola, Negri's bodies in hydrophobia, von Prowazek's bodies in trachoma, Mallory's bodies in scarlet fever, etc. Influenced, perhaps, by contemporaneous studies in malaria and other protozoal diseases, investigators have described complicated life histories based partly upon direct observations and partly upon analogy. The granules were hailed more or less tentatively as the actual parasitic causes of the diseases, were grouped among the protozoa, and were given generic and specific names, of which *Cytoryctes vacciniæ* in vaccinia, and *Neurocytes hydrophobiæ* in hydrophobia will serve as examples.

2. But closer and repeated scrutiny of the life histories did not turn out to be reassuring, and led investigators to question whether a real step had been made in advance. A reaction set in and several papers appeared showing most clearly that in some cases at least the granules are not cell parasites but deposits of pathologic nature. By common consent the protozoal terminology was abandoned and the names of the discoverers reinstated.

3. Faced by this association of more or less specific granulations with certain infectious diseases, the organisms of which have not been seen and remain almost

¹ The early literature is reviewed by Councilman, Magrath, and Brinckerhoff (1904), by Calkins (1904), by Paschen (1911), and by von Prowazek (1912).

wholly unknown, von Prowazek (1907) offered a compromise hypothesis according to which the cytoplasmic granules are of dual nature, consisting of microorganisms embedded in a substance of nucleolar origin produced by the cell in response to their action. Von Prowazek has proposed that these organisms be grouped in a new class which he has called Chlamydozoa—literally, mantle animals—to indicate this habit of being clothed in a mantle of cellular material. In his opinion they are characteristically intracellular parasites and differ from bacteria in their method of multiplication as well as in other respects. He conceived their life history to be somewhat as follows: In the stage of elementary corpuscles they are extracellular and filterable; soon after penetration through the cell membrane they may be detected in the form of tiny initial bodies. These become coated with cellular material, grow, mature, and break down with the liberation of large numbers of the original elementary corpuscles, which, in turn, enter and infect neighboring cells with a repetition of the process.

Lipschütz (1912) has devised the term Strongyloplasmata to indicate organisms endowed with much the same attributes. He groups (1921) under this heading the viruses of molluscum contagiosum, bird-pox, sheep-pox, vaccinia, scarlet fever, verruga peruviana, herpes (zoster, genitalis, and febrilis), varicella, variola, and paravaccinia, and is supported in his contention regarding vaccinia by Gins (1922) and other recent investigators.

It seems almost to have become the fashion to classify organisms about which very little is known in one or the other of these two groups. For example, during the war the ravages of typhus and trench fever demanded very close attention in both camps, which contributed directly to our knowledge of organisms which appear to be intimately concerned in the etiology not only of these two diseases but also of Rocky Mountain spotted fever and perhaps of tsutsugamushi disease, or Japanese river sickness. They have been called *Rickettsia* in honor of Ricketts who lost his life in Mexico in 1910 while investigating their behavior in typhus, but the term is unfortunate because it carries with it the suggestion that the organisms differ from ordinary bacteria and form a group exhibiting certain characteristics in common. Although it has not been possible to prove that during some phase of their life cycle they are of dual nature (consisting in part of cellular substance), they have been referred to the Chlamydozoa by da Rocha-Lima (1916), Jungmann (1919), and others.

Looking through the recent literature one finds the opinion repeatedly expressed that real advances in our knowledge of vaccinia will only be made with the help of new methods of technique.

Steinhardt and his associates (1913, 1914) and Harde (1921) have invoked the methods of tissue culture and have succeeded in obtaining a definite multiplication of the virus *in vitro* but have not been able to concentrate it greatly or to determine its relation to vaccine bodies.

What appears to be a new avenue of approach to the study of infectious diseases of unknown etiology is contributed by MacCallum and Oppenheimer (1922), who so concentrated the virus of vaccinia, through the method of differential centrifugation, that they have been able to observe directly certain granules, which may be the infective agents, under dark-field illumination and with ordinary powers of the microscope. "They occur singly or in small groups, or sometimes in tiny beaded chains. . . . They stain faintly with Löffler's methylene blue, are gram negative, do not stain with neutral red or trypan blue, stain blue or red with Wright's stain, and take a fairly deep stain with carbolfuchsin." The authors believe that: "these granules are apparently identical with those seen in tissues and smears from vaccinia and smallpox by Prowazek, Paschen, Hallenberger, and others." A similar method is advised by Ségal (1922) for concentrating the virus of typhus fever.

A large supply² of tissues from vaccinated rabbits in various stages of the reaction has given me an opportunity to experiment widely in technique and thus to make a cytologic study of the cornea with three definite objects in view; namely, (1) to trace the origin of the material of which the vaccine bodies are constituted, (2) to ascertain whether it is in part of chlamydozoal nature, and (3) to determine how closely it is related to the granules described by MacCallum and Oppenheimer in vaccine lymph.

OBSERVATIONS.

If the edge of a sharp scalpel is passed over a typical corneal lesion and the cells thus obtained are immersed in physiological salt solution and examined unstained under direct illumination, they may be seen to contain a great variety of materials some of which are not visible in control preparations of normal corneas. These may be partly resolved into the following groups.

1. Vaccine bodies, which are easily recognized by their relatively large size, low refractive index, and their tendency to be closely associated with the nuclei. Two of them are illustrated in Fig. 18, which

² This material was kindly placed at my disposal by Dr. Noguchi. It consisted of two rabbits 1 day after infection with vaccine prepared by a special method (Noguchi, H., Pure cultivation *in vivo* of vaccine virus free from bacteria, *J. Exp. Med.*, 1915, xxi, 539) by which it is freed from bacterial contamination, two 2 days after infection, three 3 days, two 4 days, one 5 days, three 6 days, one 7 days, one 8 days, one 9 days, and three controls, making nineteen in all.

should be compared with Fig. 1 representing a similar cell from an unvaccinated cornea.

2. Highly refractile spherical droplets which are usually gathered together in clumps and are probably of lipoidal nature.

3. A few scattered, rod-shaped mitochondria which may be definitely identified by the addition of a little Janus green, which colors them specifically when used in sufficient dilution (Fig. 20).

4. Large masses of coccal and bacillary shaped bodies of low refractive index, as represented in Fig. 19. Their occurrence is variable and does not seem to parallel the vaccine reaction. No independent motility could be established. They may be temporary phase granules owing their appearance to some alteration in osmotic conditions, or they may represent an accidental contamination with bacteria.

5. Large, highly refractile droplets of neutral fat which vary considerably in number, resist the solvent action of 1.5 per cent acetic acid drawn under the cover-glass, and are blackened by treatment with osmic acid.

Through the addition of brilliant cresyl blue 2 B³ to the physiological saline solution in a concentration of about 1:25,000, the vaccine bodies are almost instantly stained a color varying from pink to dark blue (Figs. 3 to 17) and the stages in their formation may easily be studied. Why von Prowazek (1912) failed to obtain a definite color reaction with this dye I am unable to suggest, unless the sample used was of different constitution or employed in a different way.

As a control measure brilliant cresyl blue was applied to testicle lesions with the same result. It was found that the same material will stain a brick-red color with neutral red and that Janus green will color it when sufficiently concentrated. Unfortunately, staining with these dyes is largely a process of selective adsorption, so that it does not convey any information regarding the chemical constitution of the material stained. But it is, perhaps, not without significance, regarding the nature of vaccine bodies, that the dyes are true solutions commonly used for the study of cellular constituents, and that they have achieved no popularity as an aid to the study of microorganisms.

³ For the manufacture of brilliant cresyl blue 2 B see Schultz (Schultz, G., Farbstofftabellen, Berlin, 1914).

Although they are relatively non-toxic (Hogue, 1922), these methods of supravital staining are open to serious question unless very carefully controlled. Since the cells are slowly dying, not through any toxic action of the stain but on account of traumatism and unfavorable environmental conditions, it is important not to prolong observations more than 30 minutes after the cells are removed from the cornea; certainly the moment that they show evidences of shrinkage, or of the adsorption of fluid with the release of molecular bombardment causing brownian movement, or of coloration of the nuclei, the preparations should be discarded.

The fragmentary appearance of the smaller vaccine bodies precludes any possibility of confusion with the segregation apparatus, originally described by Renaut, which is brilliantly colored by this technique in certain blood and connective tissue cells in which it takes the form of clusters of rounded droplets. Another point of distinction is that in smallpox similar bodies are found within the nuclei. A high development of this apparatus is suggested by Simpson (1922) as a criterion for the identification of monocytes.

With the fact in mind that in some respects the study of fixed tissues is less likely to be misleading because in them all vital activities are simultaneously arrested in some definite physiologic phase, every effort has been made to extend and to correlate both lines of inquiry. Many fixatives,⁴ stains,⁵ and microchemical methods⁶ have been applied to infected and normal cornea and testicles.⁷

⁴ Zenker's fluid with and without acetic acid, formalin, Regaud's fluid, Giemsa's sublimate mixture, acetic-sublimate, absolute alcohol, Altmann's fluid, Bensley's acetic-osmic-bichromate mixture, osmic acid in various concentrations, da Fano's modification of Cajal's silver method, etc., were used. In addition, "*Klatsch*" preparations, made as suggested by Ewing, were fixed by heat, by the vapor of formalin and osmium, by absolute alcohol, and in other ways.

⁵ The stains used were Borrel's, Giemsa's, Löffler's methylene blue, Mallory's eosin and methylene blue, Wright's stain, iron-hematoxylin, Pappenheim's pyronine and methyl green, carbolfuchsin-acid violet, aniline fuchsin-methyl green, Herxheimer's Scharlach R, etc.

⁶ Mayer's muchematein for mucus, the Macallum-Bensley iron reaction as suggested by Nicholson, Millon's reagent, and Schultze's oxidase reaction.

⁷ I am also indebted to Dr. Noguchi for allowing me to study his extensive series of stained preparations of vaccinia in rabbits and in calves.

In corneal cells supravitaly colored with brilliant cresyl blue, the irregularity in the form and distribution of the material from which the vaccine bodies are built is most noticeable. It varies from the very smallest aggregates, probably even beyond the limits of microscopic visibility, which do not concentrate the dye, and which cannot be resolved with the aid of the best lenses (Fig. 3), to roughly spherical masses upwards of $5\ \mu$ in diameter (Figs. 11, 12, and 15). This variability is exhibited by neighboring cells in almost any active stage of the process from 2 to about 9 days after vaccination. This is shown with equal clearness in fixed tissues (Figs. 21 to 45). After search through a number of preparations, or even in a single section, masses of material may be selected which correspond in size and shape to the elementary corpuscles, initial bodies, and reaction bodies described in the literature;⁸ but considered all in all, so haphazard is their morphology that it is difficult to satisfy oneself of the existence of a progressive series of morphological changes indicative of independent growth other than a simple increase in size of the aggregates through the accretion of more and more material. It is equally difficult to make out a breaking down of the large masses with a liberation of the original tiny fragments. Fig. 35 may represent a dispersal of elementary corpuscles, or it may represent a stage in the condensation of small bodies into a larger mass, depending upon which way it is interpreted.

That we are in fact dealing with a tremendous development of material which does not owe its ultimate origin to an infective process is shown by the demonstration of traces of a similar substance when the same technique is applied to the unvaccinated cornea. Its substantial similarity, as observed in normal cells supravitaly colored with brilliant cresyl blue and in normal cells after fixation in Zenker's fluid (less acetic acid) and staining with Giemsa's stain may be seen by comparing Figs. 2 and 22. There is also a close parallelism between its increase in amount, under the influence of vaccination, as one follows it in supravitaly stained cells and in fixed tissues. When colored with a relatively strong solution of Janus green

⁸ A close comparison may be drawn with Figs. 2 to 6 in the section on vaccine in von Prowazek's handbook to protozoa.

(1:10,000) the material changes to pink and bleaches with the formation of the color base and leuco base on reduction at the same time as do the other cytoplasmic constituents. After fixation in Altmann's fluid, and other mixtures containing osmium, it is, like the chromidial substance in gland cells, somewhat more diffuse. It would be unsafe to say that it occurs in definite aggregates in normal living cells *in vivo*, for the technique of its demonstration in living cells and in fixed tissues may easily, and probably does, produce artificial changes in the colloidal substratum of the cytoplasm. In other words, the material, as we see it, is the expression of the treatment we accord the cells; its occurrence in small quantities is not dependent upon vaccination.

Owing to their larger size, the vaccine bodies lend themselves more easily to microchemical analysis than does this primordial material, but beyond the statement that they are in part, at least, of protein nature, little may be said with certainty. Their resistance to tryptic and peptic digestion has been noted by von Prowazek (1912). Attempts to secure a definitely positive reaction to Millon's reagent and to the Bensley-Macallum test for iron have been unavailing, but my failure may be due to technical difficulties. Neither do they respond to Schultze's reaction for oxidase granules. They are almost immediately dissolved when a 1.5 per cent solution of acetic acid is drawn under the cover-glass. Since vaccine bodies which are apparently similar develop in the interstitial cells of the testis, and are recorded in the literature in many other locations, it follows that the original material is to be regarded as a fundamental constituent of protoplasm.

The phagocytosis of entire leucocytes and of other corneal cells is a phenomenon of common occurrence (Figs. 16, 39, and 40), but I think that the vaccine bodies do not arise through the digestion of red blood cells,—an old idea which Woodcock (1921) has recently reaffirmed,—because I have not been able to distinguish any traces of hemoglobin coloration in freshly teased corneal cells. The test for masked iron, on which he places so much reliance, can hardly be expected to differentiate between iron-containing compounds of hematogenous and nuclear origin. Moreover, the properties of the bodies, as detailed above, and the occurrence of minute deposits of similar material in unvaccinated corneas are not easily reconciled with this view. The

theory that they are formed through the phagocytosis of leucocytic fragments breaks down in the absence of data regarding consecutive stages in the process. In the normal cornea there is little or no breaking down of leucocytes, and after vaccination the leucocytic increase is somewhat variable and does not closely parallel the development of definitive vaccine bodies. Neither are vaccine bodies more abundant, or larger, near foci of leucocytic destruction.

It seems more likely that the material of which they are composed is endogenous. That it is not related to mitochondria is evident from the application of the Janus green reaction (Fig. 20) and from the general appearance of mitochondria in fixed preparations of the cornea (Fig. 45). Although I have not been successful in obtaining satisfactory slides of reticular material (Golgi apparatus) in the cornea, silver preparations of vaccinated testicles indicate that it does not constitute a source of confusion. In consideration of Ewing's (1904-05) studies with "*Klatsch*" preparations, a nuclear origin is strongly indicated, some investigators going so far as to suggest that in studying vaccine bodies we are dealing wholly or in part with extruded nucleoli. The avidity with which the vaccine bodies take up basic dyes and their topographic association with the nuclei are worthy of comment in this connection. The failure of brilliant cresyl blue to bring to light similar material within the nuclei (Figs. 2 to 17) does not militate against this conception because the dye is not able, in dilute solution and in so short a space of time, to penetrate through the nuclear membranes. At present it seems only safe to conclude that the material may be in part of nuclear origin. It would be very difficult to prove that it is wholly so.

When, under the stimulus of vaccination, the material is deposited in fairly large masses, it shows evidences of heterogeneity in both living cells and in fixed tissues. Supravital staining with brilliant cresyl blue reveals the presence of blue-staining droplets, or granules, within the larger pink-staining masses. The blue droplets vary in size, but only in rare instances are they as large as the pink-staining material (Fig. 17). They are also of less frequent occurrence. In some cases the vaccine bodies contain masses of high refractive index and irregular shape, which do not stain and which appear to be of less fluid consistency. Occasionally these occur free in the cytoplasm.

Fixed preparations also furnish evidence of corresponding diversity in composition. Similar droplets are visible in the vaccine bodies after a variety of fixations, of which Zenker, acetic-sublimate, formalin, and Regaud may be mentioned. They remain uncolored after treatment with the Borrel combination (as suggested by Calkins) Mallory's eosin-methylene blue, carbolfuchsin-acid violet, and other dyes. The chromatophobe, refractile masses, above mentioned, have not been seen after fixation. With Wright's stain after Zenker fixation, a rough gradation may be established between small irregular vaccine bodies which are colored dark blue and certain larger ones in shades of gray which take a pink or a light robin's egg blue color, as represented in Figs. 27 and 36. Sometimes these larger masses are homogeneous, or they may be surrounded with a fringe of dark blue material, as indicated in Fig. 36. Staining with aniline methyl violet, treating with Gram's iodine solution, and differentiating in 95 per cent alcohol discloses a similar heterogeneity, as shown in Fig. 40. Giemsa's stain (Fig. 41) brings to light much the same effect, it being possible to modify at will the color values from dark blue to reddish blue by inserting traces of colophonium in the alcohol of differentiation, as suggested by Wolbach, Todd, and Palfrey (1922) for the demonstration of *Rickettsia*.

From these observations I infer that the material is labile, and that, as the larger vaccine bodies are formed, it undergoes some dissociation or takes up substances from the cytoplasm so that it loses its original optical homogeneity; but this change can hardly be regarded as suggestive of the presence in it of independent microorganisms in the hypothetical stage of initial bodies.

Neither is the physical behavior of the material suggestive of the presence in it of microorganisms. In cells which are still living, it has not been possible to detect evidence of independent motility. The smallest bodies show a tendency to be distributed fairly uniformly throughout the cytoplasm and the larger ones to be congregated near the nuclear membrane. In common with other cytoplasmic materials its form and position are determined by cellular stresses, by electrical charge, and by other physical and chemical forces. For example, in the depth of the lesion, where the cells are often pressed together laterally, the deposits of material fre-

quently assume an elongated shape and become disposed parallel to the long axis of the cell. When the shape of the cell is altered through manipulation, the shape assumed by the material also changes and in some cases it seems to flow upon the surface of the nucleus, as is shown in Figs. 5 and 9, but the movement exhibited is in no sense independent or ameboid. That the material is very fluid in consistency and exhibits but little internal organization requires no further proof. The larger masses are perhaps more fluid than the smaller, more angular fragments, and have rounded up into spheres by virtue of the law of least surfaces.

In this respect also the study of permanent preparations is confirmatory. The habit the material shows of conforming in shape to that of the cell under pressure is illustrated in Fig. 28—its flowing motion in Fig. 30. As in the living cells, so also here the larger masses are spherical, but they show another property in their tendency to lodge in infoldings of the nuclear membrane. Sometimes they appear to press into the nucleus from opposite sides (Fig. 33), while in other cases they are found in deep invaginations of its surface, which may be single or multiple, and which convey the impression, when cut in transverse section, that the vaccine bodies are actually intranuclear, as represented in Figs. 34, 38, and 40. But pictures of this kind are not so easily interpreted. Whether the vaccine bodies actively indent the nuclei by virtue of being of more solid consistency, or whether they flow into depressions produced in the yielding surfaces of the nuclei through shrinkage, it is difficult to say. It seems clear, however, that the indentations are artificial since they are not to be seen in living cells (compare Figs. 3 to 20).

Considerable attention has been devoted in the literature to the areas of clear cytoplasm, or halos, which surround the vaccine bodies (Figs. 23 to 35). Investigators have called to mind approximately similar halos as accompaniments of bacteria in tissues. The fact that they are not visible in the case of vaccine bodies supravitaly stained with brilliant cresyl blue is not very helpful, because neither do I find the bacterial halos in insect material. Nevertheless, it can hardly be denied that their appearance is strongly suggestive of shrinkage at interfaces between fluids of different consistency and composition. That their extent is in some measure proportional to the size of the

vaccine bodies will be seen by comparing Figs. 23 and 27 with Fig. 33. The presence of slight halos about the nuclei (Figs. 25 and 26), though probably produced in the same way would hardly be taken to mean that the nuclei behave like independent microorganisms.

In discussing the significance of cell inclusions in scarlet fever, Mallory (1903-04) expressed the opinion that:

"In order to prove that a series of bodies are stages in the developmental cycle of a protozoon we are dependent on three things, namely, ameboid motion, definite and characteristic morphology of the various bodies found, and the demonstration that the bodies go through a progressive series of changes or developmental cycle resulting in increase in size followed by division and the re-formation of the small bodies from which the series started."

From the foregoing account it is apparent that I have not been able to fulfill any of these conditions.

The problem is complicated by the fact that well formed vaccine bodies have been repeatedly recorded outside the cells. In some of my preparations certain bodies, bearing a resemblance, perhaps entirely superficial, to intracellular vaccine bodies, are to be seen in the fibrous tissue beneath the epithelial layer of the cornea. Whether they have been formed *in loco* or have escaped from degenerating epithelial cells or leucocytes cannot be definitely stated in the absence of specific criteria for their identification. The chlamydozoal hypothesis does not provide for their development apart from the cells. Attention has already been directed to the fact that according to this theory they are said to be of dual nature, consisting of intracellular parasites in the form of initial bodies cloaked in a mantle of material elaborated by the cell. This assertion belongs to the category of statements which are easy to make and exceedingly difficult either to prove or to disprove. If the granules described by MacCallum and Oppenheimer are in fact the infective agents, and, if they maintain throughout their life cycle the morphological and tinctorial properties ascribed to them, then it appears to be unlikely that the virus is contained within the vaccine bodies. But, should the virus be beyond the limits of visibility with the best lenses, then its relation to the vaccine bodies is not easily determined.

While I have not made a cytological study of other diseases attributed to chlamydozoal organisms, a careful review of the literature does indicate, that, as far as the nature of the visible cytoplasmic granulations is concerned, their inclusion in a single category is scarcely warranted. Particularly does this appear to be the case in trachoma (Solovief, 1921).

In comparing the properties of the granules described by MacCallum and Oppenheimer in vaccine lymph with those of typical intracellular vaccine bodies, several points of similarity and of dissimilarity may be noted. (a) Like the granules of vaccine lymph the vaccine bodies are not specifically colored with trypan blue, and are colored various shades of blue and red with Wright's stain. They apparently surpass the granules of vaccine lymph in the intensity of their coloration with Löffler's methylene blue and especially with carbolfuchsin. (b) Unlike them, they are Gram-positive in the sense that they resist decoloration with alcohol after staining with aniline methyl violet and treatment with Gram's iodine. They are, moreover, intensely stained with neutral red. But still more striking is the difference in morphology. Instead of being characterized by their uniformity, the vaccine bodies are extremely variable. They bear no resemblance to tiny beaded chains of streptococci, as do the granules in vaccine lymph described by MacCallum and Oppenheimer.

To the claim that these reactions are not specific and mean but little one way or the other,—which is freely admitted,—and, further, that one would not expect the granules in vaccine lymph to maintain their original uniformity of morphology after penetration into the cell where they find themselves under entirely different surroundings, it is but natural to advance the observation of the existence of traces of material similar to the vaccine bodies in unvaccinated corneal cells. If the granules which these investigators have described are in truth the infective agents, they cannot be taken to correspond with material seen in uninfected cells, however imperfect our knowledge of its chemical constitution may be.

Since, however, the report of MacCallum and Oppenheimer is of a preliminary nature, many important details being reserved for a subsequent contribution, it seemed desirable to obtain lymph from the

same source and to repeat their work in order to make a more direct comparison with intracellular vaccine bodies.⁹

Bearing in mind the results obtained by MacCallum and Oppenheimer, I took about 5 cc. of this lymph, centrifuged it at high speed for 1 hour, drew off the turbid supernatant fluid,—which in their experiments contained the virus,—and divided it into six portions. Through the addition of varying amounts of glycerol to samples of Locke's solution, solutions were made up to the following specific gravities, determined by hydrometer: 1.11, 1.12, 1.13, 1.14, 1.15, and 1.16. One of these fluids was added to each of the six portions and centrifuged. Although the fresh supernatant fluid was examined in each case, no aggregates suggestive of organisms were found. In attempting to prepare permanent specimens, the relatively large amount of glycerol constituted a considerable handicap. Fixation in formalin vapor and absolute alcohol proved unsuccessful, but after prolonged treatment of smears with the vapor of a slightly warmed 2 per cent solution of osmic acid followed by desiccation, it was possible to color certain granulations intensely blue with Giemsa's stain in the lymph which had been centrifuged in a fluid of 1.14 specific gravity. It will be recalled that MacCallum and Oppenheimer believe the specific gravity of the virus to be about 1.12 or 1.13.

On close examination these granules were seen to be roughly spherical and to vary in diameter from 0.2 to 0.4 μ . Sometimes the larger ones are slightly angular. They occur free or embedded in a loose coagulum which stains a light lilac color. Although they are quite frequently grouped end to end in series, the individual segments are not always of the same size and rarely exceed four or five in number. The same granules are tinted gray, not black, with osmic acid. Judging from the brief description by MacCallum and Oppenheimer their appearance is only remotely suggestive of the more uniform bodies which they found.

⁹ I am again indebted to Dr. Noguchi for his kindness in giving me a large supply of vaccine lymph which he obtained from the H. K. Mulford Laboratories through the courtesy of Dr. W. F. Elgin.

CONCLUSIONS.

Vaccine bodies in living corneal cells may be specifically stained by the addition of a small quantity of brilliant cresyl blue 2 B to the physiological salt solution in which they are being observed. Their appearance by this method (Figs. 3 to 17) corresponds with that seen in fixed preparations (Figs. 22 to 42). Both lines of study reveal the existence of traces of similar material in unvaccinated corneal cells. As this increases in amount during the reaction, it behaves like an integral, cytoplasmic constituent of fluid consistency and shows no evidence of being endowed with any measure of independent vitality. The low grade of structural differentiation which it does exhibit, in living cells as well as in fixed tissues, is not suggestive of the presence within it of independent microorganisms. The material differs radically in its morphology and microchemical reactions from the granules observed by MacCallum and Oppenheimer in vaccine lymph.

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EXPLANATION OF PLATES.

PLATE 58.

The figures have been drawn with a Zeiss apochromatic objective of 1.5 mm., compensating ocular 6, and camera lucida at the table level, giving a magnification of 1,500 diameters. All represent living corneal cells in physiological saline solution viewed by direct illumination. Figs. 1, 18, and 19 are unstained cells. Fig. 20 is a cell supravitaly stained with Janus green B and the remainder are of cells stained with brilliant cresyl blue 2 B. The corpuscles of Guarnieri, or vaccine bodies, were colored pink and blue, while the nuclei remained quite unstained.

FIG. 1. Living, unvaccinated, and unstained corneal cell in which rod-like mitochondria and other granular material may be distinguished.

FIG. 2. Cell from the same animal, supravitaly stained with brilliant cresyl blue, showing traces of the material of which the vaccine bodies are built.

FIG. 3. Cell 4 days after vaccination, supravitaly stained in the same way, showing a larger amount of the same material.

FIG. 4. Cell 5 days after vaccination similarly treated. The material shows signs of heterogeneity in the form of clear unstained vacuoles.

FIG. 5. Cell 4 days after vaccination, similarly stained, showing the spread along the surface of the nucleus of a vaccine body, in which it is possible to distinguish materials of different staining reaction.

FIG. 6. Cell from the same cornea, stained in the same way, containing a larger vaccine body, exhibiting both unstained vacuoles and blue-stained granules.

FIG. 7. Cell 2 days after vaccination similarly stained.

FIG. 8. Cell 5 days after vaccination, stained in the same way and containing a heavy deposit of material.

FIG. 9. Cell 2 days after vaccination stained in the same way.

FIG. 10. Cell 1 day after vaccination, containing several small heterogeneous vaccine bodies.

FIG. 11. Cell 5 days after vaccination, with a very heavy deposit of material.

FIG. 12. Cell 5 days after vaccination, with a large solitary vaccine body.

FIG. 13. Cell 2 days after vaccination, containing a solitary vaccine body in which large and small droplets may be distinguished, which took the blue stain.

FIG. 14. Cell 5 days after vaccination, containing several vaccine bodies with droplets (or granules) which took a blue coloration.

FIG. 15. Cell 5 days after vaccination, containing a very large solitary and apparently homogeneous vaccine body.

FIG. 16. One corneal cell phagocytosed by another in a 5 day lesion.

FIG. 17. Cell from the same cornea, containing a vaccine body in two parts staining differently.

FIG. 18. Unstained corneal cell 7 days after vaccination, containing two large and slightly refractile vaccine bodies closely applied to the nuclear membrane. The finer granulations may be resolved into indistinct rod-like mitochondria and a few highly refractile droplets of lipid-like material.

FIG. 19. Corneal cell from the same lesion, also unstained. It contains a mass of coccal and bacillary bodies which is only very occasionally met with.

FIG. 20. Cell from a 7 day vaccination, supravitaly stained with Janus green to demonstrate more sharply the mitochondria. It contains a vaccine body as large as the clear unstained nucleus.

PLATE 59.

These figures have been drawn under the same magnification. All of them represent fixed and stained preparations. At least two factors are concerned in their small size, as compared with Figs. 1 to 20: first, the shrinkage resulting from the technique employed, and second, the tendency of the living cells illustrated in Figs. 1 to 20 to become slightly flattened, thus increasing their apparent size, but not their actual volume.

FIG. 21. Normal corneal cell from a control animal fixed in Zenker's fluid and colored with Giemsa's stain. The irregular deposits of material in the cytoplasm are colored dark blue in the original preparation. On infection this gives rise to typical vaccine bodies.

FIG. 22. Cell about 2 mm. from the lesion in a cornea 1 day after vaccination, fixed in Zenker's fluid without acetic acid and colored with Giemsa's stain. The same irregular material is again colored dark blue.

FIGS. 23 and 24. Cells 5 days after vaccination, fixed and stained in the same way, showing an increase in the material giving rise to vaccine bodies.

FIG. 25. Cell 6 days after vaccination, fixed and stained in the same way, representing very high development of the small bodies.

FIG. 26. Cell 5 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain, illustrating the formation of larger vaccine bodies.

FIG. 27. Cell from a lesion of 6 days, fixed in Zenker's fluid and colored with Wright's stain, containing a large vaccine body stained light blue (robin's egg color), partly surrounded by a mantle of dark blue material, and other vaccine bodies disposed just beneath the cell membrane.

FIG. 28. Cell 4 days after vaccination, fixed in Zenker's fluid and stained with carbolfuchsin and acid violet which shows up the vaccine bodies in crimson against a light green background. It indicates an accommodation in the shape and position of the vaccine bodies to that of the cell.

FIG. 29. Cell from a 4 day lesion, fixed in acetic-sublimate and colored with Giemsa's stain, containing a very deeply colored and homogeneous nucleus.

FIG. 30. Cell from a 5 day lesion, fixed in Zenker's fluid and colored with Giemsa's stain, showing a large vaccine body containing clear droplets of uncolored material, closely applied to the nuclear membrane.

FIG. 31. Cell 1 day after vaccination, fixed in Zenker's fluid without acetic acid and colored with Giemsa's stain, containing a solitary spherical vaccine body.

FIG. 32. Cell from the same preparation possessing a smaller single vaccine body.

FIG. 33. Cell 6 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain, showing the hour-glass-like indentation of the nucleus by the vaccine bodies.

FIG. 34. Cell 6 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain, showing vaccine bodies occupying depressions in the nuclear membrane.

FIG. 35. Cell 2 days after vaccination, fixed in Giemsa's sublimate mixture and colored with Giemsa's stain, showing solitary and accessory vaccine bodies.

FIG. 36. Cell from a lesion of 6 days, fixed in Zenker's fluid and colored with Wright's stain, containing a large vaccine body stained light blue (robin's egg tint), with an irregular envelope of very dark purplish blue material.

FIG. 37. Cell 5 days after vaccination, representing the pocket-like indentation of the nucleus.

FIG. 38. Cell from the same preparation as Fig. 34.

FIG. 39. One corneal cell phagocytosed by another containing well developed vaccine bodies, 5 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain.

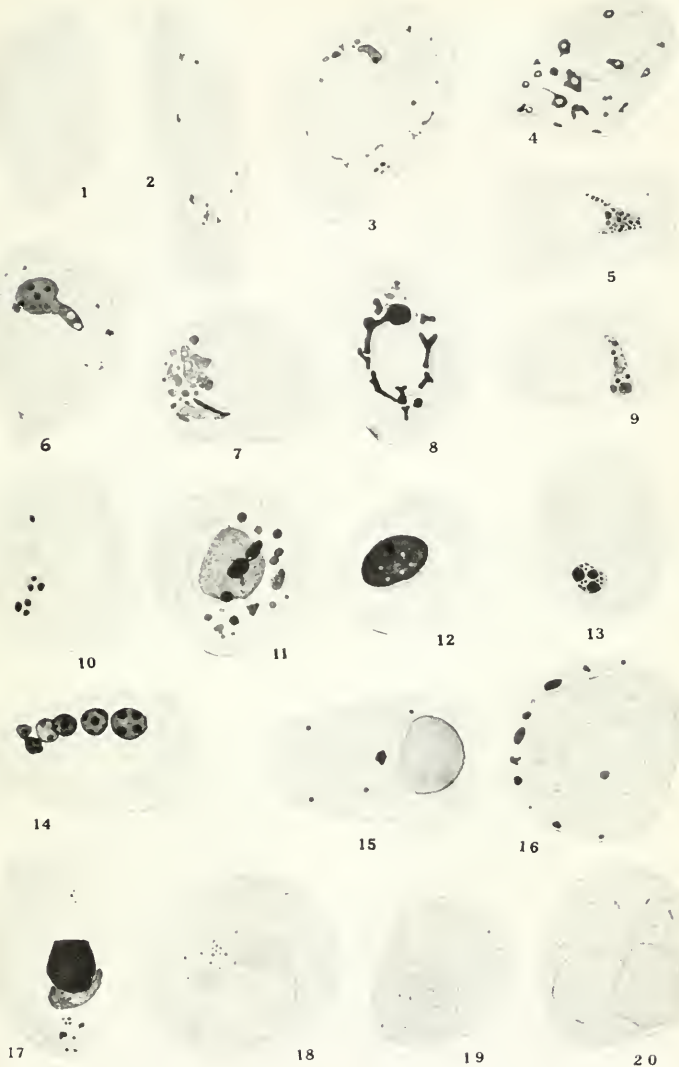
FIG. 40. A similar act in phagocytosis, in which both cells contain vaccine bodies, from a cornea 6 days after vaccination, fixed in Zenker's fluid, stained with methyl violet, treated with Gram's solution, and decolorized in 95 per cent alcohol. The vaccine bodies retain the stain much more strongly than do the nuclei. One of the bodies in the phagocytosed cell is made up of both Gram-positive and Gram-negative material.

FIG. 41. Cell from a cornea 5 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain, containing a large vaccine body, the center of which was colored dark blue and the periphery light blue.

FIG. 42. Cell from the same preparation as Fig. 35, illustrating also nuclear infoldings and halos.

FIGS. 43 and 44. Constricted and binucleated cells from the same specimen as Fig. 41.

FIG. 45. Cell distant about 4 mm. from the lesion in a cornea 3 days after vaccination, fixed in Altmann's fluid and stained with fuchsin and methyl green. The mitochondria are rod-like and filamentous. They stained bright red in sharp contrast to the homogeneous green-colored cytoplasm in which the material destined to form the vaccine bodies is not visible.



(Cowdry: Supravital staining of vaccine bodies.)



(Cowdry: Supravital staining of vaccine bodies.)

EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

X. THE IMMUNIZING EFFECTS IN RABBITS OF SUBCUTANEOUS INJECTIONS OF KILLED CULTURES OF *BACTERIUM PNEUMOSINTES*.

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It is evident from the results of the serological experiments described in Papers VII¹ and IX² of these studies that the reaction of the animal body to intratracheal and intravenous injections of living *Bacterium pneumosintes* derived from the nasopharyngeal secretions of influenza patients involves the production of serum antibodies. Evidence of the protective effects of such injections against subsequent infection with *Bacterium pneumosintes* has also been reported.^{2,3}

It appears, therefore, that *Bacterium pneumosintes* has the antigenic character common to bacteria in general, and that the mechanism of protection against it is similar to that which comes into play in the case of infections with other pathogenic microorganisms. If this is so, it follows that subcutaneous injections of killed cultures of *Bacterium pneumosintes* may be expected to induce a similar protective reaction. Recently, we have had the opportunity to put this hypothesis to experimental test. A study of the serological and immunological reactions of rabbits to prophylactic subcutaneous injections of killed *Bacterium pneumosintes* is reported in the present paper.

The opportunity for such a study was afforded by the recurrence of epidemic influenza in New York City in the early months of 1922. This recurrence provided us with four new strains of *Bacterium*

¹ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 553.

² Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxvi, 501.

³ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 1.

pneumosintes which had not undergone prolonged artificial cultivation and were, therefore, pathogenic for rabbits.² With these the protective effects of subcutaneous injections could be tested, both against subsequent infection of the lungs with *Bacterium pneumosintes*, and, as a corollary, against the secondary invasion of the lungs by other bacteria. The study also furnished evidence of the local and general reactions which might be expected to follow the subcutaneous injection of killed cultures of *Bacterium pneumosintes*, and thus prepared the way for similar observations in man.

Methods.

Preparation of the Killed Cultures of Bacterium pneumosintes.—The experimental evidence ^{1,2,3} points to the antigenic identity of all of our strains of *B. pneumosintes*, whether derived from the epidemic of 1918-19, the recurrence of 1920, or the recent recurrence of 1922. It did not seem advisable, therefore, to prepare a polyvalent vaccine of all the available strains, but, in order to include possible antigenic differences, which the serological tests did not disclose, two cultures were chosen and used in equal quantities,—Strain 17, isolated in 1918 directly from the nasopharyngeal secretions of an influenza patient, and Strain 34, obtained in February, 1922, from lung tissue derived from a rabbit in the second passage of the active agent from Case 34.

The organisms were grown for 3 to 5 days in 100 cc. Florence flasks of *B. coli* broth,² seeded with 1 cc. of a dextrose broth-rabbit kidney culture of Strain 17 or 34. After centrifugation the supernatant medium was decanted and the sediment suspended in 0.85 per cent salt solution and heated for 30 minutes at 56-60°C. The suspension was then standardized on an opacity scale and tested for sterility.

Standardization of the Suspensions.—Because of wide differences in the size of bacteria, the standardization of suspensions by counting methods does not afford comparable data on the amount of bacterial substance present, especially since the accepted error in counting methods is often as great as 25 per cent. On the other hand, it is convenient to speak of bacterial suspensions in terms of the bacterial count. What is lacking is a comparative standard to which bacteria of various sizes can be referred.

We have adopted *Staphylococcus albus* as a convenient standard and have correlated the bacterial count and the opacity of its suspensions in a number of tests. Using the Wright method for counting and the depth of disappearance readings described by one of us (Gates⁴), it was found that 1,000 million heat-killed staphylococci corresponded to a corrected opacity of 3.47 cm.

⁴ Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.

Regardless of the actual number of *B. pneumosintes* in a given suspension, we have standardized the killed cultures on the staphylococcus scale. For example, a suspension of *B. pneumosintes* which has a corrected opacity of 3.47 cm. is said to contain $\frac{1,000 \text{ million}}{\text{S. E.}}$ organisms (Staphylococcus Equivalent). In other words, the amount of bacterial substance present is assumed to correspond approximately to the amount contained in a suspension of 1,000 million staphylococci per cc. Suspensions of *B. pneumosintes*, standardized by this method, have been checked by comparison with equivalent quantities of *Staphylococcus albus*, *B. typhosus*, and Meningococcus Type A in Hopkins tubes.⁵ The amounts of bacterial sediment obtained corresponded within the limit of observation of the tubes.

Procedure.—Twenty-six apparently healthy adult rabbits were given three subcutaneous injections of heat-killed *Bacterium pneumosintes*, at intervals of 5 days. With the Staphylococcus Equivalent as a measure, the doses chosen were $\frac{2,000 \text{ million}}{\text{S. E.}}$, $\frac{4,000 \text{ million}}{\text{S. E.}}$, and $\frac{4,000 \text{ million}}{\text{S. E.}}$ *Bacterium pneumosintes*, a total for each animal of $\frac{10,000 \text{ million}}{\text{S. E.}}$. The injected suspensions contained equal quantities

of Strains 17 and 34, in 1 to 2 cc. of physiological saline solution. In order to localize the subcutaneous reaction, the injections were made through a shaved area, cleansed with soap and water and alcohol, on the most dependent portion of the abdomen.

Reactions.—None of the rabbits showed any significant local reactions or rise in temperature following the first injection of $\frac{2,000 \text{ million}}{\text{S. E.}}$ organisms. After the second injection $\left(\frac{4,000 \text{ million}}{\text{S. E.}}\right)$ most of the animals showed a mild, local reaction, consisting of redness, heat, and swelling which persisted for 2 days. The third injection rarely caused local symptoms. No general symptoms referable to the inoculations were noted. One rabbit was found dead on the 2nd day after the third injection, and two others were found to be suffering from an infection prevalent among the stock rabbits, and were killed.

⁵ Hopkins, J. G., *J. Am. Med. Assn.*, 1913, lx, 1615.

Four other rabbits were given the total dose of $\frac{10,000 \text{ million}}{\text{S. E.}}$

killed culture of *Bacterium pneumosintes* in a single subcutaneous injection. Two of these rabbits developed localized areas of heat, redness, and swelling. The other two showed practically no local reaction. Blood counts made on these animals before and after injection showed no immediate change in the total leucocyte count, or in the proportion of mononuclear cells, such as is characteristic of infection with the living organisms. The body temperatures were not affected by the injection.

Agglutinin Formation.

From 10 to 32 days after the third subcutaneous injection, nineteen of the rabbits were used in the protection experiments to be described. The blood serum of fifteen of them was tested for the presence of specific agglutinins, 10 to 27 days after the final injection. Of these sera, four failed to agglutinate the homologous strains of *Bacterium pneumosintes* in the lowest dilution tested, 1:4. Five sera, in this dilution, showed agglutinins against one or both strains. Five other sera agglutinated the strains when diluted 1:8, and one in a dilution of 1:16. In view of the individual variations in response among rabbits, and the relatively low agglutinogenic properties of *Bacterium pneumosintes*² these results are what would be expected. They indicate that the subcutaneous injection of killed cultures of *Bacterium pneumosintes* induces antibody formation. But the critical test of immunization is not the titer of serologically demonstrable antibodies, but protection against subsequent infection.

As a preliminary to the series of protection experiments with the subcutaneously inoculated animals, the four rabbits that were injected with a single dose of $\frac{10,000 \text{ million}}{\text{S. E.}}$ organisms were tested for

lowered resistance on the day following inoculation. Attention has repeatedly been called to one of the characteristic features of infection with *Bacterium pneumosintes*; namely, the loss of resistance to secondary infection which occurs in the injured lung.^{6,7} In

⁶ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 373.

⁷ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiv, 1.

view of the nature of the lesions caused by *Bacterium pneumosintes* in the pulmonary tissues, and the localization in these damaged tissues of other bacteria, injected intravenously, it is natural to suppose that it is the local disorganization and destruction of the lung structure that opens the way to secondary infections. Nevertheless, it was conceivable that *Bacterium pneumosintes* might have some inherent, specific property of promoting infections by other organisms and it seemed important to know whether, in the absence of a pulmonary lesion, the presence of *pneumosintes* protein in the body might reduce the natural resistance to other bacteria during what is commonly called a negative phase.

To test this possibility, two of the four rabbits that received 10,000 million
S. E. killed organisms in one dose were injected intravenously

on the following day with a dose of atypical Type II pneumococcus which was harmless to normal animals but infective for those suffering from a pulmonary lesion induced by *Bacterium pneumosintes*.

One of the animals remained well, gave negative blood cultures on the 2nd day, and, when killed for autopsy on that day, showed no lesions of the lungs, cultures of which remained sterile. The other rabbit died on the day following injection. No obvious lesions were found. The blood culture, post mortem, yielded the bacillus of rabbit septicemia. This infection, from which two other vaccinated rabbits were found to be suffering, was prevalent among the stock. Since the subcutaneous injection of killed cultures of *Bacterium pneumosintes* apparently did not lower the resistance of the two animals to the pneumococcus, these accidental infections presumably bear no relation to the problem in hand.

The third rabbit was similarly injected with a non-infective dose of *Streptococcus hemolyticus*, and the fourth with *Bacillus pfeifferi*. Neither streptococcus nor Pfeiffer's bacillus could subsequently be recovered by blood culture, and at autopsy the organs of the rabbits, including the lungs, appeared normal. Cultures of the lung tissue were negative.

Protection Experiments.

Experiments to test the protective efficacy of the subcutaneous injections of killed cultures were divided into three groups.

The vaccinated rabbits in Group A were injected intratracheally with the active agent of rabbit passages in the 1922 series, in doses that proved infective for normal control animals. The vaccinated rabbits in Group B were intratracheally injected with mass cultures of pathogenic (1922) strains of *Bacterium pneumosintes*. The vaccinated rabbits in Group C were injected intratracheally with *Bacterium pneumosintes*, and then intravenously with other bacteria, in doses which, following a similar intratracheal injection of *Bacterium pneumosintes*, caused pneumonia in uninoculated controls. Typical protocols may be cited to illustrate each group.

Group A. To Test the Resistance of Vaccinated Rabbits to Pathogenic Doses of the Active Agent of Rabbit Passages. Experiment 1.—Rabbit A (vaccinated): Last subcutaneous injection Apr. 29, 1922. May 9. Leucocytes 5,000, of which 2,200 were mononuclear cells. Temperature 39.3°C. May 10. Injected intratracheally with 3 cc. of a salt solution suspension of ground lung tissue derived from a rabbit in the third passage of the active agent from a recent case of early epidemic influenza (Case 28²). May 11. Leucocytes 5,200, mononuclears 3,224. Temperature 39.3°C. May 12. Leucocytes 9,000, mononuclears 5,670. Killed. Lungs and other organs show no lesions. Aerobic and anaerobic cultures of lung tissue yielded no growth.

Rabbit B (normal control): May 9. Leucocytes 14,400, of which 6,336 were mononuclear cells. Temperature 39.3°C. Conjunctivæ normal. May 10. Intratracheally injected with 3 cc. of the ground lung suspension given to Rabbit A. May 11. Leucocytes 11,000, mononuclears 4,840. Temperature 39.4°C. Conjunctivitis. May 12. Leucocytes 8,400, mononuclears 2,856. Temperature 39.5°C. Conjunctivitis. Killed. Only the lungs showed lesions. Both lungs were the site of a hemorrhagic edema and an emphysema, without consolidation. Microscopically the affected tissues showed marked edema of the interalveolar strands with a cellular exudate of mononuclear and polymorphonuclear cells. The lumina of the bronchi were filled with serum, leucocytes, and cells from the necrotic and exfoliated epithelium. Among the cilia of the bronchial epithelium clumps of minute bacteria, morphologically similar to *B. pneumosintes*, were noted. Anaerobic, as well as aerobic cultures, failed to yield a growth, however. A repetition of this experiment gave the same results.

The experiments illustrated by these typical instances show that subcutaneous injections of killed cultures of *Bacterium pneumosintes* cause the development of an effective resistance to pathogenic doses of the active agent derived from the nasopharyngeal secretions of influenza patients. The identity of this active agent with *Bacterium*

pneumosintes has already been demonstrated. Tissue suspensions were used because it was thought that *Bacterium pneumosintes* contained in the lungs of affected rabbits after repeated passages might be enhanced in virulence and pathogenicity, and hence afford a more severe test of resistance than was to be obtained with the laboratory strains which were studied in Group B.

Group B. To Test the Resistance of Vaccinated Rabbits to Pathogenic Doses of B. pneumosintes. Experiment 2.—Rabbit A (vaccinated): Last subcutaneous injection Apr. 29, 1922. May 15. Leucocytes 7,200, of which 2,880 were mononuclear cells. Temperature 39.2°C. Conjunctivæ normal. Injected intratracheally with 3 cc. of a salt solution suspension of *B. pneumosintes*, Strain 34, representing the third generation in mass culture. May 16. Leucocytes 8,000, mononuclears 4,160. Temperature 39.2°C. Conjunctivæ normal. May 17. Leucocytes 7,200, mononuclears 4,176. No change in temperature. Conjunctivæ normal. Killed. No lesions found in the lungs or other organs. Aerobic and anaerobic cultures of the lung tissue remained sterile.

Rabbit B (normal control): May 15. Leucocytes 12,000, of which 5,760 were mononuclear cells. Temperature 38.5°C. Conjunctivæ normal. Injected precisely as was Rabbit A. May 16. Leucocytes 10,000, mononuclears 4,400. Temperature 39.5°C. Conjunctivitis. May 17. Leucocytes 6,000, mononuclears 2,700. Temperature 39.6°C. Killed. The lungs showed the hemorrhagic edema, emphysema, cellular reaction, and bronchial lesions already described as characteristic of the effects of *B. pneumosintes*.⁸ Among the cilia of the bronchial epithelium and in the interalveolar tissues, numerous clumps of minute microorganisms, morphologically identical with *B. pneumosintes*, were found. Aerobic cultures of the lung tissue showed no growth; anaerobic cultures yielded *B. pneumosintes*.

This experiment is typical of the five in Group B. In spite of the fact that a large dose of a pathogenic strain of *Bacterium pneumosintes* was employed, the protection afforded by the previous subcutaneous injections of killed culture was sufficient to prevent invasion of the lung.

Since the intratracheal injections of *Bacterium pneumosintes* failed to infect and to produce pathological lesions in the lungs of the vaccinated rabbits in Groups A and B, these animals presumably would have shown a normal resistance to pulmonary infection with other bacteria. The rôle of secondary infections in human influenza is so

⁸ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

important, however, and the reduction of pulmonary resistance induced by *Bacterium pneumosintes* in experimental animals is so characteristic and significant, that we were led to test the resistance of vaccinated rabbits to other bacteria, following intratracheal injections of *pneumosintes* cultures. These experiments comprise Group C of the present series and were carried out with an atypical Type II pneumococcus, a hemolytic streptococcus, and *Bacillus pfeifferi*.

In this group of experiments the procedure described in Paper V of this series⁷ was employed. First, cultures of atypical Type II pneumococcus, *Streptococcus hemolyticus*, and *Bacillus pfeifferi* were injected intravenously into normal rabbits and a non-infective dose was determined in each case. Then the pathogenicity of mass cultures of the 1922 strains of *Bacterium pneumosintes*, injected intratracheally into normal rabbits, was confirmed (Control Rabbit B, Group B). Finally the animals previously prepared by vaccination, and normal controls, were injected intratracheally with the tested mass cultures of *Bacterium pneumosintes*, and on the following day each was injected intravenously with the dose of one of the organisms above mentioned that had proved non-infective for normal rabbits. The results of these experiments are illustrated by the following protocols.

Group C. To Test the Resistance of Vaccinated Rabbits to Infection with Other Bacteria Following the Intratracheal Injection of B. pneumosintes. Experiment 3. B. pneumosintes and the Pneumococcus.—Rabbit A (vaccinated): Last injection May 2, 1922. May 16. Leucocytes 9,300, of which 2,140 were mononuclears. Temperature 39.8°C. Injected intratracheally with 3 cc. of a mass culture of *B. pneumosintes* similar to that used in Experiment 2. May 17. Leucocytes 11,400, mononuclears 4,450. Temperature 39.9°C. Injected intravenously with 3 million atypical Type II pneumococci, suspended in 1 cc. of saline solution. (This was the tested non-infective dose for this culture of pneumococcus.) May 18. Leucocytes 7,600, mononuclears 3,570. Temperature 40.1°C. May 19. Leucocytes 12,000, mononuclears 5,760. Temperature 40.2°C. May 20. Leucocytes 8,600, mononuclears 4,730. Temperature 40°C. Blood taken for culture yielded no growth. Killed. Lungs and other organs appeared normal. Aerobic and anaerobic cultures of the lung tissue remained sterile.

Rabbit B (normal control): May 16. Leucocytes 6,300, of which 3,090 were mononuclears. Temperature 39.2°C. Conjunctivæ normal. Injected intratracheally with *B. pneumosintes*, as was Rabbit A. May 17. Leucocytes 6,000, mononuclears 1,200. Temperature 39.5°C. Conjunctivitis. Injected intra-

venously with 3 million pneumococci, as was Rabbit A. May 18. Leucocytes 5,600, mononuclears 2,520. Temperature 41.2°C. Conjunctivitis. May 19. Leucocytes 18,800, mononuclears 7,520, polymorphonuclears 11,280. Temperature 40.7°C. Conjunctivitis. Blood culture made on this day yielded the pneumococcus. Killed. Lungs voluminous with edema and emphysema and showed numerous hemorrhages. Lower right lobe dark, solid, and resistant to section. Fibropurulent discharge from bronchi. Microscopically, hemorrhage, edema, and emphysema were noted in some areas. Other areas were the site of a peribronchial consolidation and polymorphonuclear exudate. Impression smears of the cut lung showed numerous polymorphonuclear cells, and free and phagocytosed pneumococci. Aerobic and anaerobic lung cultures yielded pneumococci. Diagnosis: pneumococcus septicemia and beginning pneumonic consolidation.

This experiment is one of three in which it was found that the pneumococcus, injected intravenously, following an intratracheal injection of *Bacterium pneumosintes*, produced a septicemia and pneumococcus pneumonia in control rabbits but not in rabbits previously vaccinated with killed cultures of the primary organism. One experiment was complicated by the presence of *Bacillus lepi-septicus* in the lung tissues, but not in the blood of the vaccinated animal. The lungs of this rabbit showed no pneumonic consolidation, and cultures of lung and blood were negative for the pneumococcus.

Experiment 4. B. pneumosintes and Streptococcus hemolyticus.—Rabbit A (vaccinated): Last injection May 2, 1922. May 22. Leucocytes 5,800, of which 3,070 were mononuclears. Temperature 39.7°C. Conjunctivæ normal. Injected intratracheally with 3 cc. of a saline solution suspension of a mass culture of *B. pneumosintes*, similar to that used in Experiments 2 and 3. May 23. Leucocytes 6,800, mononuclears 3,400. Temperature 39.3°C. Conjunctivæ normal. Injected intravenously with a saline solution suspension of *Streptococcus hemolyticus* containing 1 million microorganisms (the tested non-infective dose for this culture). May 24. Leucocytes 8,400, mononuclears 5,376. Temperature 39.2°C. Conjunctivæ normal. May 25. Leucocytes 6,600, mononuclears 3,498. Temperature 39.2°C. Conjunctivæ normal. Blood cultures taken on this day remained sterile. May 26. Leucocytes 6,600, mononuclears 3,894. Temperature 39.4°C. Conjunctivæ normal. Blood culture free from growth. Killed. Lungs and other organs appeared normal. Aerobic and anaerobic cultures of the lung tissue yielded no growth.

Rabbit B (normal control): May 22. Leucocytes 7,600, of which 2,812 were mononuclears. Temperature 38.4°C. Injected intratracheally with *B. pneumosintes*, precisely as was Rabbit A. May 23. Leucocytes 7,000, mononuclears 1,680. Temperature 39.5°C. Conjunctivitis. Injected intravenously with

1 million *Streptococcus hemolyticus*. May 24. Leucocytes 8,000, mononuclears 2,240. Temperature 38.9°C. Conjunctivitis. May 25. Leucocytes 5,400, mononuclears 3,240. Temperature 39°C. Conjunctivæ normal. Blood culture positive for *Streptococcus hemolyticus*. May 26. Leucocytes 6,800, mononuclears 1,700. Temperature 39.4°C. Conjunctivæ normal. Blood culture yielded *Streptococcus hemolyticus*. Killed. Lungs showed diffuse consolidation of contiguous parts of left upper and lower lobes (red hepatization). Remainder of lung voluminous, edematous, and emphysematous, and showed a number of small hemorrhages on surface and on section. An impression smear showed numerous polymorphonuclear cells and a few streptococci. Cultures of the lung yielded *Streptococcus hemolyticus*. Diagnosis: streptococcus septicemia and pneumonia.

Two other similar experiments with *Bacterium pneumosintes* and *Streptococcus hemolyticus* gave similar results. As in the experiments with the pneumococcus, the previous vaccination of the experimental rabbits with killed cultures of *Bacterium pneumosintes* protected the animals against pathogenic doses of the living organisms; and in the absence of a pulmonary lesion, due to *Bacterium pneumosintes*, they maintained their normal resistance to secondary infection with the streptococcus.

Experiment 5. B. pneumosintes and B. Pfeifferi.—Rabbit A (vaccinated): Last injection May 27, 1922. June 6. Leucocytes 9,000, of which 4,320 were mononuclears. Temperature 39.7°C. Injected intratracheally with 3 cc. of a saline solution suspension of a mass culture of *B. pneumosintes*. June 7. Leucocytes 11,600, mononuclears 4,872. Temperature 39.5°C. Conjunctivæ normal. Injected intravenously with 7.5 billion *B. Pfeifferi* (the tested non-infective dose of this culture) in 1 cc. of saline solution. June 8. Leucocytes 12,800, mononuclears 6,400. Temperature 39.5°C. Conjunctivæ normal. Blood culture remained sterile. June 9. Leucocytes 9,000, mononuclears 5,040. Temperature unchanged. Conjunctivæ normal. Blood cultures yielded no growth. Killed. The lungs and other organs appeared normal. Aerobic and anaerobic cultures showed no growth.

Rabbit B (normal control): June 6. Leucocytes 14,000, of which 7,420 were mononuclears. Temperature 39.2°C. Conjunctivæ normal. Injected intratracheally with *B. pneumosintes*, precisely as was Rabbit A. June 7. Leucocytes 12,800, mononuclears 3,200. Temperature 39.6°C. Conjunctivitis. Injected intravenously with 7.5 billion *B. Pfeifferi*. June 8. Leucocytes 44,000, of which 5,300 were mononuclears and 38,700 were polymorphonuclear cells. Temperature 39.9°C. Conjunctivitis. Blood culture yielded *B. Pfeifferi* in pure culture. June 9. Leucocytes 22,800, mononuclears 4,788, polymorphonuclears 18,012. Temperature 39.9°C. Conjunctivitis. Blood culture positive for *B. Pfeifferi*.

Killed. Right lower lobe of lungs dark, congested, resistant to section, with a purulent exudate in the bronchi. Histological examination revealed edema, hemorrhages, emphysema, and peribronchial and lobular consolidation. The solid areas contained many polymorphonuclear cells, with some mononuclears and respiratory epithelium in a matrix of serum and fibrin. In some areas the bronchi were filled with leucocytes, serum, and fibrin, surrounded by a necrotic and exfoliated epithelium. Impression smears showed many polymorphonuclear cells and intra- and extracellular Gram-negative bacilli, resembling *B. Pfeifferi*. Anaerobic cultures of the lung were negative, although minute organisms, resembling *B. pneumosintes*, were seen in the ciliary epithelium of affected bronchi. Diagnosis: septicemia and pneumonia, due to *B. Pfeifferi*.

Six similar experiments were made in this series. The results of four of these corresponded to those illustrated above. In the other two experiments the subcutaneous injections failed to protect the animals, and the intratracheal injection of live *Bacterium pneumosintes*, followed by the intravenous injection of *Bacillus Pfeifferi* induced a pulmonary infection to which the rabbits succumbed.

The failure of the vaccine to protect in these two instances may be attributed to individual susceptibility on the part of the rabbits, or possibly to the rather arbitrary dose of *Bacterium pneumosintes* employed. In each experiment the suspension of *Bacterium pneumosintes* for intratracheal injection was divided between the vaccinated and the control animals, 3 cc. to each, but in experiments with different cultures the potentialities of the dose employed may well have varied. Since the control animals in every instance were infected, it is certain that at least one infective dose was given in all, but in some experiments the rabbits may have received several multiples of this minimum. Thus the experiments may have been much more severe tests of the protection afforded by subcutaneous vaccination than the protocols indicate, and it was to be expected that in some instances protection would be incomplete.

SUMMARY AND CONCLUSIONS.

A series of rabbits was subcutaneously injected with three measured doses of killed cultures of two strains of *Bacterium pneumosintes* derived from the nasopharyngeal secretions of influenza patients. These rabbits were subsequently tested for the development of serum

antibodies and for the presence of an induced immunity to the living organisms, with the following results.

The serum of eleven of fifteen rabbits, tested from 10 to 27 days after the final subcutaneous injection, specifically agglutinated *Bacterium pneumosintes*, whereas normal rabbit serum did not.

Nineteen vaccinated rabbits were subjected to protection experiments. Two of them were unaffected by an intratracheal injection of *Bacterium pneumosintes*, contained in the lung tissues of previously infected animals, in a dose which typically affected the control rabbits. Fifteen of the other seventeen proved to be completely resistant when tested by intratracheal injections of *Bacterium pneumosintes* cultures that produced typical infections in the controls. Ten of these fifteen rabbits were injected intravenously with living cultures of pneumococcus, *Streptococcus hæmolyticus*, or *Bacillus pfeifferi* in doses which were non-infective under normal conditions, but infective, as experience has shown, in the presence of a primary lesion caused by *Bacterium pneumosintes*. In none of these animals did infection develop. The two remaining rabbits of the seventeen were not protected against *Bacterium pneumosintes* by the vaccination, and they further developed a secondary pulmonary infection with *Bacillus pfeifferi* after its intravenous injection. Control rabbits similarly injected intratracheally with *Bacterium pneumosintes*, and then intravenously with the pneumococcus, streptococcus, or *Bacillus pfeifferi* in doses that had proved non-infective for normal rabbits, uniformly developed a secondary infection with these organisms.

The mildness of the local reactions and the absence of general signs, following vaccination with *Bacterium pneumosintes*, indicate that similar injections would be well tolerated in man. There is no evidence that the subcutaneous injection of large doses of the heat-killed organisms reduces the resistance of the animal body to infections with other bacteria. In single rabbit experiments the resistance to intravenously injected pneumococci, streptococci, or *Bacillus pfeifferi* has been found unreduced immediately after vaccination with *Bacterium pneumosintes*.

STUDIES ON THE DISEASE OF GUINEA PIGS DUE TO BACILLUS ABORTUS.

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HISTORY.

That a characteristic disease of guinea pigs could be produced by inoculation with cultures of *Bacillus abortus* was first pointed out by Smith and Fabyan (1912). A more detailed study of the disease was made by Fabyan (1912). Since that time the disease has become well known to workers in animal pathology, a great many of whom have utilized the susceptibility of the guinea pig to infection with this organism as a means of detecting it in animal tissues and discharges.

The observations on this disease which are recorded in this paper are largely on the pathogenicity of the organism and the resistance of the animal to infection. The data were collected preliminary to, and in connection with an experiment to determine the value of heat-killed cultures of the organism in producing an active immunity to *Bacillus abortus*, the results of which are reported in one of the accompanying papers (Hagan, 1922).

The Minimum Infective Dose.

Since it is a well recognized fact that most immunities are but relative and may be overwhelmed by a large amount of infection, though perhaps functioning perfectly for a reasonable amount, an attempt was made, in connection with the immunization experiment just mentioned, to determine the minimum amount of culture needed to produce infection regularly.

Search of the literature failed to show any data on this point. Smith and Fabyan had used comparatively large amounts of material in their work. Smil-

lie (1918) had found, in one experiment, that a standard suspension, which he had been using in his work, could be diluted at least 64 times without altering appreciably the disease process in his guinea pigs. Further than this he had not gone, but it was evident that he had not nearly approached the minimum.

The strain of *B. abortus* used in this work was isolated from the placenta of a cow (No. 898) by passage through a guinea pig. The culture had subsequently been passed through a second guinea pig immediately before use. A plain veal infusion agar slant was inoculated with a culture from the spleen of the latter animal, the tube sealed, and incubated for 48 hours at 37°C. The resultant growth was washed from the surface of the slant with a small amount of physiological salt solution, making a uniform suspension. After a little shaking this was diluted with more salt solution to make a standard density which was determined by the method of Gates (1920). A reading of 2.4 cm. was selected arbitrarily as an end-point. Such a suspension is somewhat denser than a 24 hour typhoid culture in bouillon. Hereafter in this paper such a suspension will be referred to as a standard.

From this suspension, dilutions were made by transferring 1 cc. portions from one tube to the next in series, each tube containing 9 cc. of salt solution. Thus a series of dilutions was made as follows: 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000, 1:1,000,000, 1:10,000,000, and 1:100,000,000. Intermediate dilutions of 1:5,000,000 and 1:50,000,000 were also made.

Preliminary work had shown that 1:100,000 dilutions of such suspensions were capable of infecting, and consequently higher dilutions were used in this series. Five guinea pigs, males, varying in weight from 355 to 410 gm. and of approximately the same age (Group V) were injected intraperitoneally with 1 cc. portions of the last five dilutions respectively. Infections resulted in the animals receiving dilutions of 1:1,000,000, 1:10,000,000, and 1:100,000,000; while the 1:5,000,000 and 1:50,000,000 failed to infect. Infection was determined by testing for the development of agglutinins and eventually, by killing the animals, observing lesions, and recovering *Bacillus abortus* culturally.

Infection by so minute a quantity of material as a 1:100,000,000 dilution of a standard suspension was a surprise. For confirmation, a second series of animals was tested with the same dilutions as before. Five male animals weighing from 415 to 470 gm. (Group V₂) received a 24 hour culture of the same strain as before. Just previous to use the strain had been recovered from another guinea pig at autopsy. In this case, infection of all animals occurred.

It was now evident that still higher dilutions of the suspension were necessary in order to approach the minimum infective dose. A third series of animals (Group V₃), consisting of eight animals weighing between 385 and 495 gm., was selected. The culture was of the same strain as before but had just been recovered from the heart's blood of a white mouse which had died within 24 hours after being inoculated with the strain. Before inoculation into the mouse the strain had been recovered from the spleen of a guinea pig which had been affected with a very severe form of the disease. The standard suspension was made up as before from a 24 hour culture, and from this in turn dilutions to 1:100,000,000, 1:200,000,000, 1:500,000,000, and 1:1,000,000,000. Two animals were injected with each of these dilutions. All became infected.

For the determination of the number of *Bacillus abortus* in the suspensions used for inoculation, the plating method described in one of the accompanying papers (Hagan, 1922) was used.

The degree of accuracy of this method is not known, as it was difficult to determine because of the small size of the organism. When several plates are made from one suspension, however, the discrepancy in the bacterial count of the various plates generally does not exceed 30 or 40 per cent, and frequently there is less than 10 per cent difference. It is advisable to add a drop of sterile blood to the surface of the agar plate at the time the suspension is added, so that the two will mix while spreading. The viscosity of the blood aids in making the film uniform over the surface, and somewhat higher counts result from the enrichment of the medium.

Although duplicate counts on a single suspension were reasonably consistent, it was found difficult to make up two suspensions which would give approximately the same count when using the Gates disappearing loop as a gauge. Thus four suspensions, standardized carefully with the Gates apparatus and plated in the same way, though at different times, gave the following counts: (a) 592, (b) 423, (c) 1,030, and (d) 1,451.

These figures represent the average counts of from two to four plates containing 0.1 cc. of a 1:1,000,000 dilution of the respective standards. The variation, amounting to over 300 per cent in the case of the extremes, must be due, in part at least, to inaccuracies in the method or operation of the opacity gauge by which the suspensions were standardized, although there is a large source of error in the pipetting when making up the dilutions. However, in estimating the number of bacteria injected into the guinea pigs, these discrepancies of count become of little consequence because of the great dilutions worked with.

If the counts given above may be accepted as representative of the true count of the living organisms in the suspensions, their average, which amounts to 874, should represent a mean which is within 100 per cent, one way or the other, of the true count in other suspensions made up in the same manner. In other words, a suspension of *B. abortus* made up in salt solution from the surface of a 24 hour slant agar culture, and having a reading of 2.4 cm. on the Gates gauge, has a mean calculated count of living organisms amounting to 8.74 billions per cc. with a minimum of about 4.5 billions and a maximum of about 13.0 billions. Since the guinea pigs were inoculated with dilutions running into the millions, these variations are reduced immediately to a few hundred organisms, at the most.

TABLE I.

Guinea pig No.	Dilution of standard suspension.	Computed No. of organisms injected.	Result.
Group V.			
2059	1:1,000,000	8,740	+
2060	1:5,000,000	1,750	-
2058	1:10,000,000	874	+
2056	1:50,000,000	174	-
2055	1:100,000,000	87	+
Group V ₂ .			
2172	1:1,000,000	8,740	+
2176	1:5,000,000	1,750	+
2179	1:10,000,000	874	+
2177	1:50,000,000	174	+
2175	1:100,000,000	87	+
Group V ₃ .			
2232	1:100,000,000	87	+
2233	1:100,000,000	87	+
2230	1:200,000,000	44	+
2231	1:200,000,000	44	+
2228	1:500,000,000	18	+
2229	1:500,000,000	18	+
2227	1:1,000,000,000	9	+
2226	1:1,000,000,000	9	+

The minimum infective dose of *Bacillus abortus* for guinea pigs has not been determined, since infection was produced in the majority of the animals with the smallest amounts of material used. The computed number of bacteria used in the experiments is given in Table I.

Many workers who have been using guinea pig inoculations for diagnostic purposes have found it possible thus to detect, at times, *Bacillus abortus*, in material from which cultures had failed, the presumption being that sparsity of the organisms accounted for the differences in results. Grounds for believing in a rather high infectivity of the organism for the guinea pig have existed, therefore. This belief is amply substantiated by the data presented above. As a means of detecting *Bacillus abortus*, guinea pig inoculations are highly efficient.

Individual Variations in Susceptibility.

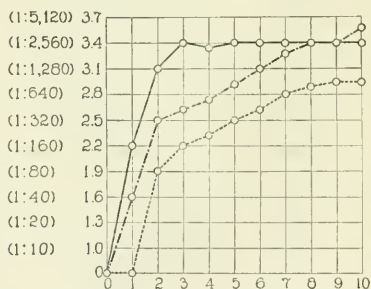
The failure of Guinea Pigs 2060 and 2056 (Table I, Group V) to become infected, although given from two to twenty times as much material as was needed to infect other animals, indicates a considerable degree of immunity in these animals. The resistance of No. 2056 was not tested subsequently, but No. 2060 was given a second inoculation at the time, and with the same material with which Group V₃ was infected (Table I). The dosage in this case was 1 cc. of a 1:1,000,000 dilution, and although this was from 100 to 1,000 times as great as that used in the animals of Group V₃, the response to the inoculation was much more tardy than that of any of the animals of the group mentioned. Although No. 2060 proved to be the most refractory of many animals tested, infection was accomplished by the second inoculation, which was of less than 10,000 organisms. Smillie reports two cases in which cultures were positive in certain parts of the alimentary tract of a bovine fetus, whereas the guinea pig inoculations from the same material gave negative results. While it is always possible that unequal distribution of the bacilli may produce such results, it is much more probable that resistant animals such as the two guinea pigs mentioned were used. The resistance would be effective only if very few organisms were present in the material injected.

The Effect of Variations in the Dosage on the Character of the Disease.

To determine the effects of dosage on the character of the resulting disease, a series of animals was inoculated, one-third with a heavy dose, another with a medium, and the other with a very small dose.

TABLE II.

Guinea pig No.	Inoculating dose.	Killed.	Spleen weight.	Spleen.	
				Organisms per gm.	Organisms per spleen.
		wks.	gm.		
2160	Undiluted.	1	2.18	269,700,000	587,946,000
2164	1:10,000	1	0.67	51,000	34,000
2165	1:1,000,000	1	0.63	450	280
2158	Undiluted.	4	1.02	2,600,000	2,650,000
2161	1:10,000	4	0.65	25,000	16,000
2166	1:1,000,000	4	0.69	106,000	73,000
2167	Undiluted.	6	1.39	24,000	34,000
2163	1:10,000	6	2.4	?	?
2157	1:1,000,000	6	1.67	39,000	65,000
2159	Undiluted.	9	3.02	48,000	145,000
2162	1:10,000	9	3.9	25,000	100,000
2168	1:1,000,000	9	1.13	1,400	2,500

TEXT-FIG. 1. Composite agglutinin curves of animals receiving different doses of *B. abortus*.

—○— Guinea Pigs 2158, 2160, 2167, and 2159. Standard suspension, undiluted.

○- - - - -○ Guinea Pigs 2161, 2163, 2164, and 2162. Dilutions of standard suspension, 1:10,000.

○.....○ Guinea Pigs 2157, 2165, 2166, and 2168. Dilutions of standard suspension, 1:1,000,000.

Abscissæ represent weeks, ordinates logarithms of the dilutions. Blood dilutions are in parenthesis.

A standard suspension was used for the first, a 1:10,000 dilution of this for the second, and a 1:1,000,000 dilution for the third. Four guinea pigs were inoculated intraperitoneally with each of the suspensions. All animals were bled weekly and from each group one was killed at intervals of 1, 4, 6, and 9 weeks after the inoculation. The principal results are given in Table II and Text-fig. 1.

The principal effect of changing the amount of the infecting dose is to change the time relations. A very heavy inoculation calls forth early agglutinin production and early lesions, smaller doses produce nearly the same effect but at later periods; and the smaller the dose, the longer is this period. The factor of individual variations in resistance enters rather prominently into such data as those of Table II.

The Course of the Disease.

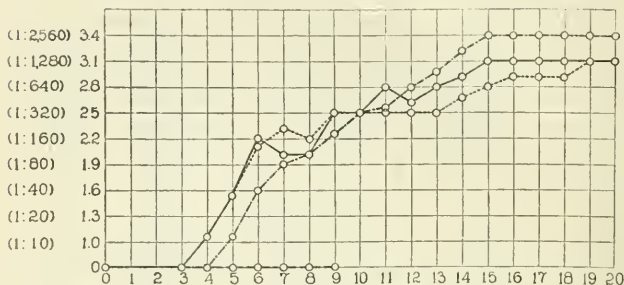
It was stated by Fabyan that the disease in guinea pigs produced by *Bacillus abortus* tended toward final recovery, although many animals might die from exhaustion and complications. These conclusions agree well with my own observations. In both his and my experiments the organism studied was isolated from cattle. Whether this is also true when the organism is isolated from other animals is a question upon which I have no data.

Inspection of all of the agglutinin curves in this and accompanying papers, except Text-fig. 2 of the present one, shows that a fall in the titer begins about the 10th week of the infection.

Two groups aggregating ten animals, the data on which are incomplete, were kept for a period exceeding 6 months after inoculation with a moderate dose of living culture of *B. abortus*. In all cases, the titer was at its height at the time of bleeding at the end of the 11th week. At the next bleeding, during the 16th week, the titer had fallen in all but one animal from the height previously occupied. During the 19th week all titers had fallen perceptibly, and at subsequent bleedings up to the time of death they were found to be falling still, slowly but definitely. When the animals were killed, after more than 6 months, only a few colonies of *B. abortus* developed in cultures from the spleen and in most cases the other organs gave no growth. It is apparent that the organisms were gradually being killed off. No animals have been kept much longer than 6 months, so that the ultimate termination of the disease is not known.

The agglutinin curves in Text-fig. 2 are exceptions to the general rule because the amount of culture used to infect was so very near the

minimum infective dose that the resultant disease was slow of onset and slow of progress. Whereas, at about the 10th week with a moderate dosage the disease would have reached its climax, in this case with a very small dose the disease was still actively progressive.



TEXT-FIG. 2. Agglutination curves of Group V guinea pigs.

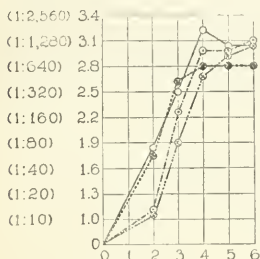
- Guinea Pig 2059.
- - -○ Guinea Pig 2058.
- ...○ Guinea Pig 2055.
- · - ·○ Guinea Pigs 2056 and 2060.

The Allcation of Culture Virulence.

All of the work reported in this paper was done with a single strain of *Bacillus abortus* isolated less than 6 months previously. A comparison of Text-figs. 2 and 3, however, will show that enormous differences in virulence existed at different times. This difference in virulence is not simply attenuation due to *in vitro* cultivation, for Text-fig. 2, in which the virulence seems to be low, illustrates the effects of a younger cultural generation of the organism than does Text-fig. 3. This variation in virulence at different times was always encountered when dealing with small doses, so that the results in one series of animals could never be exactly duplicated in another series. It was clearly due to the influence of the bodies of the guinea pigs through which the culture had been passed.

For instance, the group represented in Text-fig. 2 was inoculated with a culture which had just been passed through Guinea Pig 1958. This animal had been

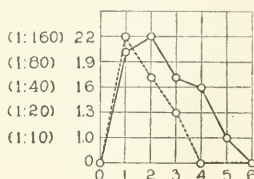
inoculated 8 weeks previous to sacrifice with a 1:1,000 dilution of a standard suspension. Only moderately severe lesions had developed, showing that the animal had been quite resistant to the infecting organism. Text-fig. 3 represents the findings in a group of animals which had been inoculated with smaller doses than those of Text-fig. 2; but the culture had just been recovered from the heart's blood of a mouse which had died within 24 hours after infection. Tracing one generation further back, it is found that the mouse was inoculated with the strain immediately after that had been recovered from the spleen of a guinea pig killed 1 week after inoculation with a large dose of culture, and that this animal had



TEXT-FIG. 3.

TEXT-FIG. 3. Agglutination curves of Group V₃ guinea pigs.

- Guinea Pigs 2233 and 2232.
- Guinea Pigs 2230 and 2231.
- - - -○ Guinea Pigs 2228 and 2229.
- Guinea Pigs 2226 and 2229.



TEXT-FIG. 4.

TEXT-FIG. 4. Agglutination curves of guinea pigs receiving a single injection of killed *B. abortus* culture.

- Guinea Pig 2118.
- Guinea Pig 2051.

exhibited marked lesions, especially great splenic enlargement. The organism thus had been passed through two animals immediately before use in the series. This treatment had apparently exalted the virulence to a very considerable degree. A third group of animals serves to demonstrate the same point. This group (Table I, Group V₂) was inoculated with the same amounts of suspension as in Group V. The agglutination curve, however, instead of resembling that of Group V (Text-fig. 2), was very steep, resembling that for Group V₃ (Text-fig. 3). In this case the culture had just been recovered from a guinea pig which had been inoculated with a large dose of *B. abortus* and killed in 1 week.

The Agglutinin Production of Bacillus abortus for Guinea Pigs.

Normal guinea pig serum will cause clumping of suspensions of *Bacillus abortus* only when in very low dilutions. Clumping in a dilution as high as 1:10 may be considered as evidence of artificial stimulation. This is easily accomplished by the inoculation of either living or dead organisms, but the former are much the more efficient.

Text-fig. 4 represents the agglutinin response of two normal guinea pigs to heavy doses of killed *B. abortus* suspension injected intraperitoneally. Comparison with Text-fig. 1 shows that the initial response is approximately the same as with heavy doses of living suspensions. After the 1st week, however, the curve falls, in the case of the dead culture, and within a few weeks has reached normal. If additional injections of dead culture are given, the response is slight and a titer of about 1:640 appears to be near the limit of their stimulation capabilities.

The administration of living cultures in quantities great enough to infect causes the agglutinin titer to rise to a height of 1:1,280 to 1:5,120, depending on the individual. Partial reactions in a dilution of 1:10,240 have been obtained in the case of a few individuals but this is quite rare.

Individual variation in the form of the agglutinin curves is very great, so that it cannot be predicted accurately. In general, however, the larger the initial dose the steeper is the first part of the curve. The ultimate height reached is the same, irrespective of the dose used.

Permeability of the guinea pig placenta to *Bacillus abortus* antibodies was demonstrated in two cases by the detection of agglutinins in the blood of fetuses taken from the uteri of infected mothers, the tissues of the fetus proving sterile upon adequate culture. In one case the titer of the mother was 1:1,280 and that of her fetus 1:160; in the other, that of the mother was 1:2,560, and that of the fetus 1:80. The fetal fluids were negative in a dilution of 1:20 in each case.

Natural Infection of Guinea Pigs with Bacillus abortus.

In the course of this work many guinea pigs, some normal and some infected, were kept together in large pens, and not a single instance of natural infection of the normal animals occurred. The animals were always of the same sex, as the two sexes were kept separate to prevent breeding during the experiment. A number of infected females left over from another experiment were divided into

two groups of five animals each and each group penned with a normal male. After 4 months, one group was killed. Four of the females were pregnant and the male was healthy. In the other group, agglutination tests at the end of the 3rd month indicated developing infection in the male. The group was sacrificed at the end of the 4th month and the following facts noted. There were no gross lesions to indicate the disease in the male. Cultures from the spleen yielded a few colonies, and those from the right epididymis showed heavy growths of *Bacillus abortus*. Cultures from other organs gave no growth. The agglutinin titer at death was 1:320. Three of the females were pregnant. Of the other two females, one was interesting as being the probable source of infection of the male animal. Aside from the usual lesions found in advanced cases of the disease (the animal had been inoculated about 6 months previously), there were two abscesses located in the wall of the uterus, one of which was discharging into its lumen. The left uterine horn was bound down by adhesions to surrounding tissues. The abscesses were old, thick walled, and filled with a thick, yellowish white, non-odorous pus, such as is frequently seen in epididymal abscesses.

It is not possible to say in what manner the male guinea pig became infected, but the finding of a *Bacillus abortus* abscess opening into the female generative organs and the isolation of the organism from the generative organs of the male, which animal showed little evidence of the usual systemic disease, forms a formidable chain of circumstantial evidence. Infection by ingestion is improbable in the light of past experience, and is rendered more so in this particular case by the fact that normal females had been associated for months with this same group of animals without contracting infection.

The Susceptibility of Guinea Pigs to Infection by Bacillus abortus through Ingestion.

The common experience that guinea pigs infected with *Bacillus abortus* may be kept with normal animals of their own sex with little or no danger of infecting the latter suggested that normal animals are probably not highly susceptible to feeding infection. This point was tested in the following experiment.

A standard suspension of *B. abortus* was made up and from this dilutions of 1:10, 1:100, and 1:1,000. With a small bulb pipette 1 cc. of each suspension was placed directly into the mouth of young normal animals. No difficulty was experienced in getting the animals to swallow the entire amount, so the exact dosage is known for each animal.

The two animals receiving the undiluted and the 1:10 dilution of the standard suspension became infected, the other two given the higher dilutions remained well. Agglutinin production in the infected animals did not become evident until the 3rd week following infection, a lag seen among inoculated animals only in those receiving a very minute dose.

It appears from this experiment that the susceptibility of guinea pigs to feeding infection with *Bacillus abortus* is actually quite low, as had been surmised. It is computed that the ratio between the minimum infecting dose of *Bacillus abortus* by inoculation and by feeding is at least as great as 1 to 100 million.

SUMMARY AND CONCLUSIONS.

1. The infectivity of *Bacillus abortus* for guinea pigs is extremely great. It is computed that somewhat less than 100 organisms are required to infect most animals.

2. Occasional animals are encountered having much more resistance to *Bacillus abortus* infection than is possessed by the average. The most resistant animal encountered was infected by a number of organisms computed to be less than 10,000.

3. The principal effect on the character of the *Bacillus abortus* disease, caused by varying the size of the infecting dose, is a change in the time relations. Very small doses produce an infection of slower course than do larger doses, but the eventual results are practically the same.

4. Judging by the agglutination curves, guinea pigs inoculated with large or medium sized doses of *Bacillus abortus* generally begin to gain mastery over the disease about the 10th week after inoculation. At this time the agglutination curve begins to decline. As was first shown by Smillie, the numbers of bacteria in the body organs begin to decline at about the 4th week but the change is rather slow until about the 10th or 12th week when a rapid decline begins, coincidently with the fall in the agglutinin titer. When the inoculating dose is small

the course of the disease may be much prolonged. The defensive forces of the body would appear to be quite inactive until a certain stage in the disease is reached.

5. The virulence of *Bacillus abortus* cultures for guinea pigs may be raised or lowered by appropriate passage through these animals.

6. Normal guinea pig blood will not agglutinate *Bacillus abortus* in a dilution of 1:10. Immune agglutinins are easily produced by inoculations with dead or living organisms. Agglutinins for *Bacillus abortus* are capable of passing the placental filter.

7. There appears to be little or no danger of normal guinea pigs becoming infected by association with diseased animals of their own sex. One instance is reported, however, of the infection of a male animal that had cohabited with infected females.

8. The susceptibility of guinea pigs to infection through ingestion of *Bacillus abortus* is relatively slight.

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THE VALUE OF HEAT-KILLED CULTURES FOR THE PREVENTION OF THE BACILLUS ABORTUS INOCULATION DISEASE OF GUINEA PIGS.

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The great economic importance of infectious abortion in cattle and swine, particularly in cattle, has provided the stimulus for many experiments aimed at the prevention of the disease through the use of preparations made from the *Bacillus abortus* and its products. Naturally the use of killed cultures of the organism was among the first of these procedures attempted and it has, perhaps, been the method most extensively tried. In spite of this the exact immunizing value of the killed cultures is not known though it is quite generally thought at the present time to be slight. The interpretation of results of immunization experiments against infectious abortion in cattle is attended with certain inherent difficulties which have left nearly all such experiments open to question.

The disease of guinea pigs due to inoculation with *Bacillus abortus*, first described by Smith and Fabyan (1912), seemed to offer a more favorable opportunity for studying the mechanism of the process of immunity than the cattle disease, since it is not attended with the difficulties found in the latter. Such a study was taken up, therefore, not only for the light which might be cast by it on the general biology of the organism but for the possible assistance which it might render toward solving the cattle problem. It was realized, of course, that results obtained in working with one species of animal cannot safely be applied to another species, by inference, unless there is strong corroboratory evidence.

HISTORICAL.

Previous attempts to immunize guinea pigs to *B. abortus* by the use of killed cultures have been made by Ascoli (1915), and by Stafseth (1920). Stafseth found that no immunity was produced by the procedure, but his work is of little value because the size of his infecting dose was so enormous as to overwhelm any but the strongest of immunities.

Ascoli's work may be criticized similarly, although it is evident that he appreciated the importance of a reasonably small infecting dose and made an effort to attain it. His immunizing cultures were killed with ether. Both single large doses of the immunizing suspension and small repeated doses were tried. The infecting doses varied from one series of animals to another, but the smallest used was 1:100,000 of an agar slant growth. The animals were killed after periods of time varying from a few days to several months. The agglutinin titer of the blood was determined when the animals were killed, and cultures were made from the spleen. The nature and extent of the lesions apparently were not considered.

The animals previously subjected to the immunization process as well as normal animals used as controls developed agglutinins for *B. abortus* and the organism was recovered from the spleens of all. It was concluded, therefore, that the use of killed cultures of *B. abortus* was ineffectual in preventing subsequent infection by inoculation.

Plan of Experiment.

Since previous work, supported by similar observations dealing with the disease in cattle, indicated that the degree of immunity produced in the animals probably would be slight, if not nil, it was planned to carry on observations upon a quantitative basis in an effort to detect immune processes which might not be of sufficient degree to afford complete protection.

A group of twenty-four female animals was selected from the stock, uniformity of age and size being sought. All were young, half grown animals weighing in the neighborhood of 350 gm. Agglutination tests with *Bacillus abortus* antigen showed the blood of all to be negative in a dilution of 1:10. One-half of the animals (twelve) were subjected to preliminary injections of killed cultures and finally all, with the exception of two animals from each group which were preserved as controls, were inoculated with living culture. Throughout the experiment all animals were kept together in a single large pen.

Evidences of immunity in the group of animals receiving the pre-

liminary treatment were sought by comparing the group with the untreated in the following respects: (a) changes in body weight; (b) differences in the agglutinin curves; (c) extent and character of the lesions, with especial reference to the size and condition of the spleen; and (d) number of *Bacillus abortus* cultivable from the spleen.

Immunization.

The culture used throughout this work was a typical strain of *B. abortus* isolated from a bovine placenta, Case A 898, by passage through Guinea Pig 1865. It grew readily on plain veal infusion agar, provided the cultures were hermetically sealed. For antigenic material this strain was grown 48 hours on plain agar in a Blake flask which had been sealed with wax. A suspension of the resulting growth was made in physiological salt solution and heated at 60°C. for 1 hour. Both cultural and animal inoculation tests proved the suspension to have been sterilized. It was diluted to a reading of 2.4 cm. on the gauge devised by Gates (1920) and stored, without preservation, in the refrigerator until ready for use.

The twelve animals selected for immunization were given intraperitoneal injections of 1 cc. of the killed bacterial suspension weekly. The manner in which the animals were reacting to the injections was gauged by the agglutination curve. The weekly injection of rather large doses of killed bacterial suspension appeared to exert little effect on the health of the animals, although reference to Table I will show that the group did not gain in weight so well as did the untreated group. The gain in weight during the period of immunization for the treated group averaged 162 gm., while for the other group the average was 231 gm.

The curve in Text-fig. 1 is typical of the agglutinin response in all of the treated animals. After a sudden rise following the first injection there was a slow rise until an average titer of 1:640 was reached, beyond which point it appears that there is no stimulation by the use of dead cultures. After six weekly injections the process was stopped and some time was allowed to elapse before the test inoculations were made, in order to allow the body to recover its equilibrium which it was thought might have been upset by the stress of the immunizing process. At the end of the 3rd week following the last dose of killed culture, the agglutinin titers began to show evidence of falling and the time was considered ripe for inoculation with the living culture.

The Infecting Dose.

The strain of *Bacillus abortus* which had been used in the preparation of the immunizing suspension was also used for infection.

The culture had been passed through Guinea Pig 2106 just prior to use. The growth from a 24 hour sealed slant agar culture was washed off with physiological salt solution and the resulting suspension diluted to make a reading of 2.4 cm. on the Gates loop gauge. This makes a suspension somewhat denser than a 24 hour typhoid culture in bouillon. Dilutions of 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} were made from the original suspension. The last three dilutions were used for inoculating, 1 cc. being injected intraperitoneally in each case.

In the matter of dosage, it was sought, in some of the animals, to approach rather closely to the minimum amount necessary to produce infection; in others to give a moderate dose; and in a few to give what might be considered a comparatively large dose. The question of the minimum infective dosage of suspensions of *B. abortus* is discussed elsewhere (Hagan, 1922). Reference to the communication cited will show that the smallest dose administered was probably several times the amount actually needed to produce infection. The individual variation in resistance is so great that smaller doses than the minimum amount given in this experiment could not safely be depended on to infect all normal animals, although $\frac{1}{100}$ of this amount probably would have infected most guinea pigs.

The animals were killed and examined in pairs consisting of one animal from each group. Some were killed within 3 weeks after the test inoculation; others in 6, 9, and 12 weeks. All were bled regularly at weekly intervals and the agglutination curves obtained.

RESULTS.

Body Weight.—A complete weight record will be found in Table I. Analysis of this shows that during the entire experiment the average gain in body weight was approximately the same in the two groups; *i.e.*, immunized group 308 gm., non-immunized 304 gm. At the same time two animals used as controls, which were not subjected to any experimentation except periodic bleeding for agglutination work, made an average gain of 430 gm. It has already been stated that during the immunization process the treated animals were outstripped in the amount of weight gained by some 70 gm. on the average for the group. From the time of inoculation to the time of death the position was reversed, the immunized animals gaining 109.5 gm. while the non-immunized gained only 53 gm. This margin undoubtedly would have been widened if the animals had been allowed to live longer, as

is indicated by the comparatively great weight losses suffered by the non-immunized animals which were allowed to live the longest.

The Agglutination Curves.—At the time of administering the living culture, the agglutinin titer of the immunized group averaged about 1:320, while the bloods of the non-immunized were negative at a dilution of 1:10. Text-fig. 1 shows the behavior in the immunized control animals. Following the last injection of killed culture there is a gradual decline in the curve lasting some 13 weeks before it reaches the base-line. Text-figs. 2 to 4 show the curves in the inoculated animals receiving different amounts of infective material. The latter halves of these curves show that there is practically no difference in the agglutinin response to the infecting dose in the two groups, and that the size of the infecting dose has made little difference in the agglutinin response. In Text-figs. 3 and 4 the smaller doses have produced a delay in the agglutinin production, and the curve for the immunized group falls appreciably before the effect of the test inoculation becomes operative. In the case of the comparatively heavy inoculation represented by Text-fig. 2 this fall does not occur.

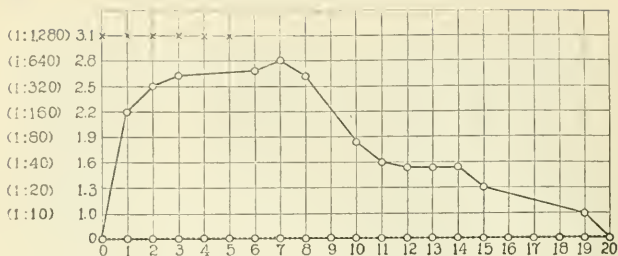
Differences in Extent and Character of Lesions.—All of the inoculated animals became infected, thus showing that the treatment was ineffectual in preventing the disease. Certain differences in the character of the disease process have been noted, however, as a result of the preliminary treatment. These have to do with the spleen, lymph glands, and kidneys.

The spleen presented the most striking lesion of the disease in all cases, immunized or non-immunized. Reference to the spleen weights in Table I shows that enlargement occurred in all cases except in Guinea Pigs 2083 and 2071, both of which were immunized animals which had received small doses of the infecting culture and were killed early. The splenic enlargement, with one exception, was greater, however, and usually much greater in the non-immunized than in the immunized group. The spleen weight for the former group averaged 4.87 gm., while for the latter it was only 3.1 gm., a difference of more than 50 per cent. There is no evidence that the character of the process in the spleen is different in the two groups. It is probable that the difference is only a matter of degree; *i.e.*, that in the immunized group the process is only delayed.

TABLE I.

Guinea pig No.	Immunized.	Injected.	Length of time lived.	Initial weight.	Maximum weight.	Weight when killed.	Loss in weight from maximum.	Change in weight during immunization.	Change in weight during disease.	Spleen weight.	Agglutinin titer when killed.	Spleen.	
												Bacteria per gm.	Bacteria per organ.
2073	+	Control.	24	410	720	720	0	+190	+100	1.07	1:40	0	0
2081	-	"	24	405	740	740	0	+215	+95	1.06	0	0	0
2074	+	10 ⁻³	24	390	670	670	0	+155	+90	4.12	1:5,120	?	?
2082	-	10 ⁻³	24	410	745	725	20	+245	+70	7.34	1:1,280	807,000	5,900,000
2083	+	10 ⁻⁶	24	370	690	690	0	+170	+110	1.07	1:160	83,000	88,000
2090	-	10 ⁻⁶	24	430	680	680	0	+190	+70	3.09	1:640	43,000	124,000
2071	+	10 ⁻⁶	24	335	710	710	0	+185	+130	1.19	1:320	615,000	731,000
2076	-	10 ⁻⁶	24	465	755	720	35	+250	-10	6.01	1:320	367,000	2,205,000
2070	+	10 ⁻⁶	42	340	645	645	0	+75	+110	3.00	1:2,560	123,000	369,000
2077	-	10 ⁻⁶	42	395	640	530	110	+200	-85	5.49	1:5,120	196,000	1,078,000
2067	+	10 ⁻⁶	42	370	660	630	30	+160	+100	2.48	1:1,280	198,000	491,000
2080	-	10 ⁻⁶	42	385	760	760	0	+235	+130	2.59	1:1,280	42,000	109,000
2086	+	10 ⁻³	63	400	750	750	0	+220	+90	2.24	1:1,280	12,500	38,000
2088	-	10 ⁻⁶	63	430	1,000	1,000	0	+240	+300	2.23	1:2,560	?	?
2075	+	10 ⁻⁶	63	410	850	850	0	+180	+240	4.8	1:2,560	33,800	162,000
2079	-	10 ⁻⁶	63	305	725	725	0	+215	+180	2.74	1:5,120	?	?

2068	Control.	85	415	800	800	0	+160	+170	1.07	0	0	0
2085	"	85	385	915	915	0	+260	+235	1.07	0	0	0
2069	10^{-3}	85	335	560	480	80	+125	- 10	5.92	1:5, 120	109,000	645,000
2078	10^{-3}	85	390	710	540	170	+230	-115	8.75	1:5, 120	75,500	662,000
2072	10^{-6}	85	365	730	730	0	+185	+155	3.26	1:2, 560	11,300	36,900
2087	10^{-6}	85	390	915	670	245	+260	- 30	4.32	1:2, 560	38,000	105,000
2084	10^{-6}	85	330	600	570	30	+140	+ 80	2.97	1:2, 560	43,000	128,000
2089	10^{-6}	85	400	830	690	140	+230	+ 20	6.17	1:5, 120	58,600	361,500

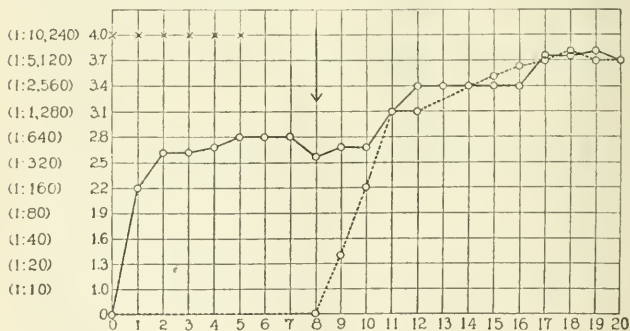


TEXT-FIG. 1. Composite agglutinin curves of normal control, and of immunized but non-infected animals.

—○— Composite curve of Guinea Pigs 2068 and 2073, animals which were immunized but not infected. The times of injecting the killed suspensions of *B. abortus* are indicated at the points x.

.....○..... Composite curve of Guinea Pigs 2081 and 2085, animals which were untreated but allowed to live throughout the experiment in the same pen with the infected animals.

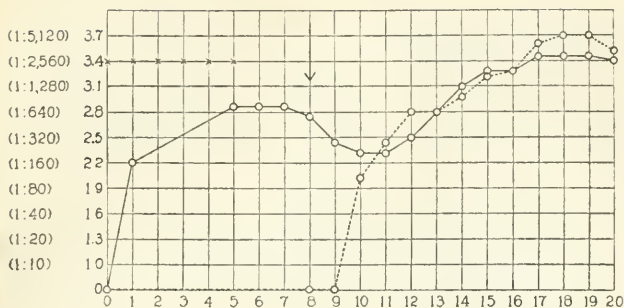
In this and all succeeding agglutinin curves, the abscissæ represent weeks, and the ordinates the logarithms of the dilution of the blood. The actual blood dilutions are given in parenthesis.



TEXT-FIG. 2. Composite agglutinin curves of immunized and non-immunized animals, infected with 1:1,000 dilution of a standard suspension of *B. abortus*. The times of injecting killed suspension of *B. abortus* are indicated by x, the time of infection by the arrow.

—○— Guinea Pigs 2068 and 2074, immunized.

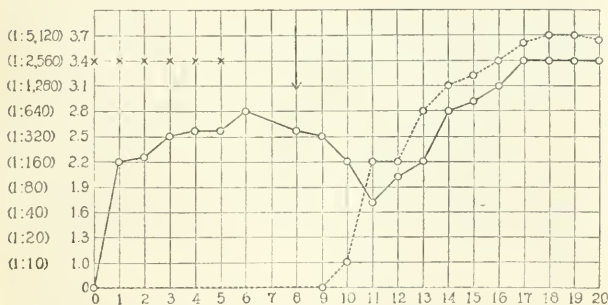
.....○..... Guinea Pigs 2078 and 2082, non-immunized.



TEXT-FIG. 3. Composite agglutinin curves of immunized and non-immunized animals, infected with a 1:100,000 dilution of a standard suspension of *B. abortus*.

—○ Guinea Pigs 2070, 2072, 2083, and 2086, immunized.

.....○ Guinea Pigs 2077, 2087, 2088, and 2090, non-immunized.



TEXT-FIG. 4. Composite agglutinin curves of immunized and non-immunized animals, infected with a 1:1,000,000 dilution of a standard suspension of *B. abortus*.

—○ Guinea Pigs 2067, 2071, 2075, and 2084, immunized.

.....○ Guinea Pigs 2076, 2079, 2080, and 2089, non-immunized.

Enlargement of the lymph glands occurred, coincident with splenic enlargement, and was more pronounced in the non-immunized animals than in the immunized.

A striking lesion in the non-immunized animals which lived the longest and in several of the non-immunized animals which were killed early occurred in the kidneys. This lesion is frequently found in the later stages of the guinea pig disease and was described by Smith and Fabyan (1912). It consists in cellular infiltration in the early stages, followed later by sclerosis. That the lesion is of great import to the body economy is rendered probable by the fact that as the severity of the sclerosis increases emaciation of the body keeps pace. The condition is always accompanied by a very large spleen, but great enlargement of the spleen is sometimes found without accompanying kidney disease and emaciation. This kidney lesion was found among the immunized animals but once, in Guinea Pig 2069, one of the animals which received a heavy infecting dose.

The Number of Bacillus abortus Present in the Spleen.—The manner of determining the bacterial count of the spleen is described in the appendix. The counts are given in Table I. It will be noted that the bacterial count per gram of splenic tissue is greater in the immunized than in the other group, while the reverse is true when the bacterial content of the entire organ is considered. The explanation for this lies in the greater enlargement of the organ in the latter group. Since this enlargement is largely due to engorgement of the sinuses with blood, the bacterial concentration per unit of volume is decreased by dilution. Taking the entire organ, however, there is an appreciably greater number of cultivable *Bacillus abortus* present in the spleens of the non-immunized than in those of the immunized animals.

SUMMARY AND CONCLUSIONS.

From the data presented it seems permissible to draw the following conclusions.

1. Guinea pigs cannot be rendered immune to the *Bacillus abortus* inoculation disease by treatment with heat-killed cultures of this organism. This agrees with the work of Ascoli, and with the general conclusions of many that dead cultures confer very little or no immunity to infectious abortion in cattle.

2. The progress of the disease can be delayed appreciably by such treatment. This is supported by the following observations: (a) the loss in body weight due to the disease is delayed; (b) the development of splenic enlargement is partially inhibited or delayed; (c) the development of renal lesions is delayed or prevented; and (d) the rapidity of multiplication of *Bacillus abortus* in the splenic pulp is reduced.

APPENDIX.

Technique of Bleeding Guinea Pigs for Agglutination Tests.—The repeated bleeding of guinea pigs for periodic agglutination tests has been accomplished in an easy and fully satisfactory manner. Many animals have been bled ten to twenty times without injury and without difficulty in obtaining the requisite amount of blood for macroscopic tube tests. The method is as follows:

The laboratory should be warm so as to encourage the circulation in the peripheral vessels. One of the ears is rubbed briskly with a pledget of cotton soaked in xylol, and then dried with a clean bit of cotton; the blood vessels are examined and one of them nicked transversely with the point of a scalpel. The vessels can be seen to better advantage if some form of illumination below the ear is used. A vigorous flow of blood generally wells up from the small incision. The blood is taken into a 0.1 cc. serological pipette which is provided, for convenience, with a length of rubber tubing and a mouthpiece. One pipetteful (0.1 cc.) is immediately delivered into 0.9 cc. of citrated physiological salt solution, making a dilution of the blood of approximately 1:10. If necessary to work with low dilutions, two pipettefuls (0.2 cc.) may be delivered into 0.8 cc. of salt solution, making a dilution of 1:5. The tubes are shaken up and placed in the refrigerator over night. The next day as much as 0.7 to 0.8 cc. of a perfectly clear dilution of plasma may be pipetted off.¹ Since only 0.5 cc. is needed for the agglutination tests, the quantity is ample. The citrate-salt solution consists of 0.9 per cent sodium chloride and 2.0 per cent sodium citrate in distilled water. This amount of citrate

¹ The dilution figures in this and accompanying papers are given in terms of the whole blood. The serum or plasma dilutions may be roughly computed as twice as great as that of the whole blood.

does not have any effect on the agglutinin titer so far as could be determined by checking against sera obtained by defibrination. The citrated plasma has the advantage over most sera in that it is perfectly clear and untinged with hemoglobin.

Agglutination Technique.—The technique of carrying out the agglutination tests is practically that described by Smillie, Little, and Florence (1919).

The antigen has been freshly prepared and no preservative added, although equally good results appeared to be obtained by using an antigen preserved with 0.25 per cent formalin and kept stored in the refrigerator for several months. Antigen preserved with 0.5 per cent phenol is not satisfactory because it induces a permanent cloudiness of the suspension which renders the reading of partial reactions difficult.

The Determination of the Approximate Numbers of Bacillus abortus Present in the Spleen.

Preparation of Splenic Tissue for Plating.—The following method was used for obtaining a measured and representative sample of splenic tissue for plating purposes.

A portion of the organ representing from 0.5 to 1.5 gm. is removed aseptically to a sterile weighed potato tube and its weight determined. The fragment is then crushed thoroughly in a manner to be described and diluted with nine times its weight of physiological salt solution. After thorough mixing, 0.1 cc. portions are used for plating, unless it is suspected that the bacterial count will be greater than 100,000 organisms per gm. of tissue, in which case 0.1 cc. of a 1:5 or 1:10 dilution of the original suspension is used.

For grinding or crushing the splenic tissue efficient crushers were made from heavy walled culture tubes in the following manner.

Tubes of as nearly perfect form as possible are selected from the stock and arranged in pairs, one tube of which is of such size that it will just slip easily into the other like the plunger of a syringe. The smaller tube is then constricted in its middle part by heating in a blast flame and drawing it out. The larger tube is shortened by cutting it off at such length that its mouth will be about at the level of the middle of the constriction of the smaller tube when the latter is placed within it. The inner tube is used as a pestle while the outer serves as

a mortar. The surface of the pestle is roughened by rotating it in contact with a piece of carborundum paper held in the hand. The inside of the mortar has not been altered although it probably could be improved somewhat by etching with hydrofluoric acid. Great roughening is not to be desired since the mortar is apt to be cut and broken, in the process of grinding, by sharp edges on the pestle. For the same reason abrasives such as fine sand or carborundum powder cannot be used. The apparatus is intended for crushing purposes rather than for grinding.

The crusher is wrapped in paper and sterilized by hot air. The piece of weighed spleen is placed in the larger tube and forced to the bottom with the pestle. It is now compressed and broken up by rotating and exerting gentle pressure on the pestle, the soft pulp being forced up between the sides of the pestle and the inner wall of the mortar. If the fit between the two elements of the crusher is good, by the time the pulp reaches the space in the upper end of the mortar left by the constriction of the shaft of the pestle it will be thoroughly reduced to a homogeneous paste. When the crushing has been completed small portions of salt solution are pipetted into the mortar and the pestle slowly lifted while being rotated. The vacuum in the bottom of the pestle causes the fluid to pass the pestle, thereby cleansing it of the pasty pulp mass. Several repetitions of this process generally leave the pestle clean, after which it is removed and the remainder of the quantity of salt solution needed to make a 1:10 dilution added.

Plating Methods.—With *B. abortus* ordinary plating methods are useless because this organism requires a peculiar gaseous environment which is not satisfied by the free atmospheric exchange of the ordinary Petri dish. For obtaining suitable gaseous conditions for the cultivation of the *B. abortus* in plate culture, there have been available the methods of Nowak (1908) and of Huddleson (1920). Since both of these are somewhat involved, while furthermore simple hermetic sealing of the tubes had proven an entirely satisfactory method of obtaining growth in tube cultures, it was decided to try this method with plate cultures in lieu of the others. A simple and satisfactory method of sealing the plates has been devised and the results in obtaining growth of *B. abortus* have been entirely satisfactory.

Pieces of double strength window glass were cut into 12 cm. squares, and others into pieces 12 by 24 cm. in order to handle two cultures as a unit. The edges of the glass were ground off to remove sharp points and corners. These squares were used as bases upon which the halves of the Petri dishes containing the cultures were sealed. The steps in the method are as follows:

1. Paraffin of a melting point of 55–60°C. is placed in a shallow vessel and heated until it smokes. The high temperature serves to sterilize the paraffin. When it has cooled to 60–70°C., a few degrees above its melting point, it is ready for use.

2. A glass square is placed on the table and its upper surface thoroughly traversed with the Bunsen flame. This not only sterilizes the surface but serves to warm the glass.

3. The lower half of the Petri dish containing the inoculated agar is grasped, open surface downward, with the tips of the fingers and the edges dipped into the molten paraffin to a depth of 5 to 8 mm. The entire margin should not be submerged at one time, otherwise when the plate is lifted the rush of air under the edge is apt to cause spattering of the paraffin on the agar surface. This may be avoided easily by tilting the plate when submerging. Since the temperature of the glass of the Petri dish is lower than the melting point of the paraffin, a layer of the latter in a congealed state adheres to the plate when it is lifted.

4. The plate is placed on the center of the glass square and gently pressed down. Since the square is warm, and there has been no delay between the dipping of the edges of the plate and planting it on the glass surface, an excellent seal between the plate edge and the glass base is formed.

5. To assure a perfect seal, the flame of a micro burner is run quickly around the joint between plate and base. If done properly the surface only of the paraffin layer will be melted and all cracks and crevices will be filled. If too much heat is used the glass will become heated through and will cause the paraffin to spread out in a thin layer on the base, thus forming an imperfect seal, or at best a thin seal which is apt to rupture through when a partial vacuum has formed within the plate.

Even when hermetically sealed, poured plates are not satisfactory for quantitative work when dealing with *B. abortus* for the reason that the organism will not develop in anaerobic or semianaerobic conditions. The deep lying bacilli will, in most instances, fail to develop and those that do grow will produce colonies so minute that counting is difficult or impossible. Surface inoculation has been used exclusively in this work. 0.1 cc. of the splenic suspension is pipetted onto the surface of the agar plate and spread uniformly by means of a bent glass rod. This amount of fluid will spread evenly over the surface of the agar without leaving an excess of fluid. The plate is then tilted, in an inverted position, on the rim of the lid to allow the surface to dry somewhat before it is sealed.

Growth generally appears within 3 to 4 days, though sometimes not for 5 or 6 days. Within 24 hours of the time when colonies can first be seen, they will have reached a diameter of 1 mm. if there is not too much crowding. After this, growth is comparatively slow, but if very few colonies are present they may reach a diameter of from 3 to as much as 6 mm. The colonies have no tendency to spread or coalesce. Because of the discrete character of the growth as many as 1,000 colonies may grow on a single plate without evidence of interference with each other, other than that their ultimate size will not exceed 1 mm.

Generally all colonies appear at the same time and grow at about the same rate of speed, so that they have about the same size. In some instances, however, two crops of colonies may appear. In these cases the second crop will become visible 1 to 3 days after the first as minute colonies lying between the first. The second crop colonies always remain small, seldom reaching a diameter of 0.5 mm. even when they are not crowded and are subjected to prolonged incubation.

Because of this phenomenon, it is always advisable to incubate the plates for 3 or 4 days following the appearance of the first crop of colonies before making the colony count.

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THE SUSCEPTIBILITY OF MICE AND RATS TO INFECTION WITH *BACILLUS ABORTUS*.

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It was observed by Holth (1911) that an acute disease resulted when mice and rats were inoculated intraperitoneally with cultures of *B. abortus*. It was stated that from 0.25 to 0.5 cc. of a serum bouillon culture in mice, and from 0.5 to 1 cc. in rats, was sufficient to produce a prostrating disease with fatal termination in from 2 to 5 days. The disease was essentially an intoxication. At autopsy the principal lesion found was a moist, reddened peritoneum with large numbers of the specific organism and a few leucocytes on its surface. Culturally, a few organisms could be found in the blood after death.

These observations were confirmed, in general, by Zwick and Zeller (1912), and by Ascoli (1915). In Ascoli's tables, it is noted that *B. abortus* was recovered culturally in several cases, presumably from the spleen, after periods of time as great as 2 months after inoculation, when because of insufficient dosage or artificial immunization death did not occur from the acute form of the disease. Ascoli does not mention the presence or absence of pathological changes in these cases. Zwick and Zeller noted an occasional acute swelling of the spleen, in addition to the changes described by Holth.

Fabyan (1912) showed that a type of disease entirely different from that just described might be produced in mice by intraperitoneal or subcutaneous injection of *B. abortus*. This disease was of a chronic nature, similar in many respects to that produced by the organism in guinea pigs. Although his dosage was rather high he does not mention having observed the acute disease previously described. It is possible that his statement that "many of the animals died from complications, frequently without apparent cause" refers to this form of the disease. 3 to 4 weeks after inoculation he found the spleens enlarged in thirteen of nineteen animals which had survived from a group of about thirty inoculated. Lesions in the spleen, lungs, liver, kidneys, lymph glands, and epididymis were found. Rats were inoculated also but no effect of the inoculations was detected.

INTRODUCTION.

The work just described made it clear that mice and rats could be infected experimentally with *Bacillus abortus* when a large amount of

infective material was employed for inoculation. Whether these animals could be infected with such small amounts as to make them useful in detecting the organism in diagnostic work with infectious abortion of cattle, and whether they were sufficiently susceptible to infection by ingestion to make the wild varieties a possible factor in the propagation and transmission of the disease among cattle were questions unsolved. To the working out of these two questions this paper is devoted.

The Susceptibility of White Mice to the Inoculation Disease.

Ten young, white, male mice of about the same size and age were selected from the stock and divided into five pairs, each pair being placed in a jar by themselves. The animals of the first pair were inoculated differently, one with a 48 hour serum bouillon culture of *Bacillus abortus*, and the other with an undiluted suspension of *Bacillus abortus* washed from a 48 hour agar slant culture. The second pair received a 10^{-3} , the third a 10^{-4} , the fourth a 10^{-5} , and the fifth a 10^{-6} dilution of this suspension. All were inoculated with 0.5 cc. of dilution, intraperitoneally. One guinea pig was inoculated with 0.5 cc. of the 10^{-6} dilution as a check on the virulence of the culture and for purposes of comparison.

The suspension above referred to was made by growing a bovine strain of *Bacillus abortus*, recently recovered from a lymph gland of Guinea Pig 2082, in a sealed tube of plain veal infusion agar for 48 hours, then making a suspension of the growth in sterile physiological salt solution, and diluting this to a reading of 2.4 cm. on the standardizing gauge of Gates (1920).

The results of the experiment are recorded in Table I. A number of the animals died early from an intercurrent pneumonia. The results sought, however, were obtained in a clear-cut manner. All of the animals became infected as evidenced by the recovery of the organism from the spleens. The cultural record for Animal C was misplaced and lost, and decomposition prevented cultural work on Animal B.

Table I shows clearly that white mice may be infected with very minute amounts of cultures of *Bacillus abortus*. The results with the guinea pig indicate that mouse susceptibility to infection with

TABLE I.
White Mice.

Mouse.	Infecting dose.	Autopsied.	Spleen culture.	Remarks.
A	0.5 cc. serum bouillon culture.	Died; 27th day.	Positive, moderate.	Died from pneumonia. <i>Klossiella</i> infection of kidneys.
B	Suspension 0.5 cc. undiluted.	" 3rd "	None made.	Badly decomposed. Died from pneumonia. No evidence of peritonitis.
C	" 0.5 " 1:1,000 dilution.	" 9th "	Record lost.	Purulent pneumonia.
D	" 0.5 " 1:1,000 "	Killed; 27th "	Positive, luxuriant.	Spleen enlarged X 3. Other organs normal. Agglutinins 1:160.
E	" 0.5 " 1:10,000 "	Died; 26th "	" moderate.	Spleen normal. Slight pneumonia. <i>Klossiella</i> infection of kidneys.
F	" 0.5 " 1:10,000 "	Killed; 27th "	" luxuriant.	Spleen enlarged X 3. Slight pneumonia. Agglutinins 1:320.
G	" 0.5 " 1:100,000 "	Died; 11th "	" " good.	Killed by fighting. Slight splenic enlargement. Slight pneumonia. <i>Klossiella</i> infection.
H	" 0.5 " 1:100,000 "	Killed; 27th "	" luxuriant.	Spleen enlarged X 3. Slight pneumonia. Other organs normal.
I	" 0.5 " 1:1,000,000 "	" 27th "	" "	Spleen normal size. Slight pneumonia. Agglutinins 1:40.
J	" 0.5 " 1:1,000,000 "	Died; 2nd "	" very few colonies.	Wounded by fighting. Very weak when inoculated. No lesions of internal organs.
Guinea Pig 2114	" 0.5 " 1:1,000,000 "	Killed; 27th "	Positive, moderate.	No lesions. Agglutinins 1:40.

this organism compares favorably with that of the latter animal. No experiments have been done with infected tissues, but there is no apparent reason why white mice should not serve successfully as inexpensive substitutes for guinea pigs in diagnostic work on infectious abortion of cattle.

The Susceptibility of Rats and Mice to Bacillus abortus by Ingestion.

The susceptibility of both wild and tame varieties of mice and rats to infection by feeding with *Bacillus abortus* was tested. Cultures and infected tissues were used.

TABLE II.

White Rats.

Rat.	Method of infection.	Material used.	Autopsied.	Spleen culture.	Agglutinins.	Remarks.
A	Feeding.	Culture.	52nd	—	—	
B	"	Tissue.	52nd	—	—	
C	Inoculation.	Culture.	23rd	+	1:40	No lesions.
D	"	" 1:100 dilution.	26th	+	1:40	" "
E	"	"	52nd	+	1:40	" "
F	Control.	—	52nd	—	—	

The cultures were salt solution suspensions of the growth removed from agar slants which had been incubated for 24 to 48 hours. Approximately 3 cc. of salt solution was used to suspend the growth from a single tube culture. The infected material was the livers of guinea pigs which had been infected with *B. abortus* and killed during the 4th week after inoculation. Although the organs of the body of the guinea pig are richest in the abortion bacilli at this time, the liver is never the seat of large numbers of the organism. The livers used for feeding had all yielded the *B. abortus* in moderate numbers.

The animals were allowed to fast for 24 hours before feeding. The culture suspensions were placed in Petri dishes and small bits of dry bread added to absorb the fluid. When offered to the hungry animals, the soaked bread was always eagerly consumed. The livers were ground to a pasty consistency in a mortar and smeared on pieces of bread. The paste was eagerly licked off the bread by the rats and taken fairly well by the mice. The day after the first feeding food was again withheld, and on the 3rd day the feeding of infected material was repeated as before and with the same materials.

The results of the feeding experiments are given in Tables II to VI inclusive. In each case several animals were inoculated subcutaneously as controls. It will be noted that all inoculated animals became infected, while all of the feeding experiments, with two ex-

TABLE III.

White Rats.

Rat.	Material fed.*	Autopsied.	Spleen culture.	Agglutinins.	Remarks.
		<i>day</i>			
A	Culture suspension, undiluted, 1 cc.	22nd	Cultures contaminated.	1:10	Died from pneumonia. Organs invaded with colon bacilli.
B	Culture suspension, undiluted, 1 cc.	29th	+	—	Very few organisms present in spleen. No lesions.
C	Suspension, 1:10 dilution, 1 cc.	29th	—	—	
D	Suspension, 1:100 dilution, 1 cc.	29th	—	—	
E	Suspension, 1:1,000 dilution, 1 cc.	29th	—	—	

* In this case the bacterial suspensions were pipetted directly into the mouths of the animals, so the exact dosage is known.

TABLE IV.

Gray Rats.

Rat.	Method of infection.	Material used.	Autopsied.	Spleen culture.	Remarks.
			<i>day</i>		
A	Feeding.	Infected tissue.	19th	—	
B	"	Culture.	19th	+	No lesions. Organism recovered from spleen.
C	Inoculation.	" suspension 1 cc.	22nd	+	No lesions. Organism recovered from spleen. Agglutinins 1:1,280.

ceptions, failed. The two exceptions, one a gray rat (*cf.* Table IV, Rat B), and one a white rat (*cf.* Table III, Rat B), received heavy culture feedings. The data on gray rats are limited because of the difficulty of procuring the animals. In Table III, however, it is

demonstrated that the white rat becomes infected by feeding, only if he receives a very heavy dose of the organism; and this is probably also true of the gray rat. The failure, in all cases, of the guinea pig tissue to infect is best explained on this basis.

TABLE V.
White Mice.

Mouse.	Method of infection.	Material used.	Autop-sied.	Spleen culture.	Agglu-tinins.	Remarks.
			day			
A	Feeding.	Infected tissue.	52nd	—	—	
B	"	Culture.	52nd	—	—	
C	"	"	28th	—	—	
D	"	"	28th	—	—	
E	Inoculation.	"	23rd	+	1:20	Enlarged spleen.
F	"	"	52nd	+	—	" "
G	"	" 1:100 dilu-tion.	26th	+	—	" "
H	Control.	—	52nd	—	—	

TABLE VI.
Gray Mice.

Mouse.	Method of infection.	Material used.	Autop-sied.	Spleen culture.	Agglu-tinins.	Remarks.
			day			
A	Feeding.	Culture.	28th	—	—	
B	"	"	28th	—	—	
C	"	"	2nd	—	—	
D	"	"	33rd	—	—	
E	"	"	33rd	—	—	
F	"	"	52nd	—	—	
G	"	Infected tissue.	52nd	—	—	
H	Inoculation.	Culture.	21st	+	1:160	Organs normal.
I	"	"	52nd	+	1:20	" "
J	"	" 1:100 dilu-tion.	52nd	+	—	" "

CONCLUSIONS.

1. White mice are highly susceptible to infection by inoculation with *Bacillus abortus*. The susceptibility appears to be as great as that of the guinea pig, and this animal probably can be substituted satisfactorily for guinea pigs in diagnostic work.

2. Both mice and rats are very refractory to feeding infection with *Bacillus abortus*. The failure to infect mice in this way was complete, but the feeding of large amounts of culture gave infection in rats. Subcutaneous inoculation resulted in infection of all the animals. The difficulty of infecting rats and mice with *Bacillus abortus* by feeding makes it very doubtful whether these animals can have any rôle in the propagation and spread of infectious abortion in cattle.

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THE ACTION OF SODIUM SALICYLATE UPON THE FORMATION OF IMMUNE BODIES.

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INTRODUCTION.

This study is one of a series to determine the action of the salicylates upon some of the manifestations of infection in the animal body. From a pharmacological view-point it is well established that the salicylates have an antipyretic and analgesic action in patients when administered in therapeutic doses. The analgesic action varies in different individuals and is apparently more powerful with some of the salicylate compounds than with others. This effect is obtained in many conditions, both in the general toxic state seen in most infections, and also in neuralgias and myalgias when indefinite pain is the chief symptom, and fever and other general manifestations of infectious intoxication are usually absent.

The antipyretic action is due to an increased loss of body heat and, as shown by Barbour (1), is evidenced almost exclusively by individuals who are suffering from infection; normal persons show little if any antipyretic effect after taking fairly large doses of the drug. Afebrile patients suffering from infections, on the other hand, show an increased heat loss after receiving comparatively small doses of the drug. It seems as though the heat-regulating center of this class of individuals is in a different state of sensitiveness to salicylates than is that of normal persons.

A third action of salicylates demonstrated almost exclusively in patients with the polyarthritis of rheumatic fever is that of an antiphlogistic agent. Following the administration of the drug in amounts just under the toxic dose, a most striking decrease of local heat, redness, and swelling, as well as of pain and tenderness in the

involved joints is usually observed; and, in addition, the tendency for the inflammation to spread to other joints is arrested. Patients suffering from gonorrheal arthritis or other joint infections in which bacteria can be demonstrated in the arthritic fluid are not benefited in this striking manner by salicylates. So often has this observation been repeated that the therapeutic test is frequently applied to differentiate the acute polyarthritis of rheumatic fever from that due to other causes. It seems fairly well established, on the other hand, that the duration of the general infection in rheumatic fever is not materially shortened by the drug, and that cardiac complications often arise even though the patients are taking amounts sufficient to keep their temperatures almost normal and to prevent arthritis recurring. From clinical observation alone it would seem, therefore, that the drug either lowered the infectivity of the etiologic agent of rheumatic fever by a direct action upon the virus or increased the ability of the body to react against the infection.

It is well known that salicylic acid and sodium salicylate have an antiseptic action *in vitro*; this action is much stronger with free salicylic acid than with its salts. It is, moreover, impossible for the free acid to occur in the tissues or body fluids in sufficient concentration to exert any marked bactericidal action. A bacteriostatic action must be considered; the drug may exist in sufficient concentration or in a state to inhibit the growth of the infectious agent, even though the virus is not completely killed. A study of this problem is now under way.

It is conceivable that the salicylates may increase the resisting power of the animal body by stimulating the formation of immune bodies; but in so far as we are able to determine very little attention has been given to this possibility.

Jacoby and Schütze (2) studied the opsonin and bacteriotropin content of the serum of normal and immune rabbits; the serum was obtained both before and 8 hours after the animals received a single dose of 2 gm. of sodium salicylate by stomach tube. Staphylococci, *B. coli*, and *B. typhosus* were used in the tests. The authors state that five out of twelve rabbits showed a distinct increase of immune bodies following salicylate treatment. Unfortunately, the details of the experiments are not given, so it is impossible to tell whether the opsonins or the bacteriotropins were increased the more.

EXPERIMENTAL.

In view of the fact that no definite etiologic agent has been demonstrated for rheumatic fever, it was thought advisable in our studies to use various members of the coccus group of bacteria as antigens to stimulate the formation of antibodies in rabbits, and to compare the amount of antibody formation in immunized animals receiving sodium salicylate with that of animals similarly immunized but untreated with salicylates. In some of the experiments living bacteria were inoculated into the rabbits, in others killed bacteria in the form of vaccines were employed. In all instances the antigens were injected intravenously. In addition, three experiments were performed to determine the effect of salicylates upon hemolysin formation.

The rabbits received the sodium salicylate in the form of a 2.5 or 5 per cent solution twice daily by stomach tube. In the earlier experiments this was combined with an equal amount of sodium bicarbonate; but later it was found that the salicylate was well tolerated without the bicarbonate so the latter was omitted. In most instances the daily dose of sodium salicylate was from 0.16 to 0.2 gm. per kilo of body weight; the weight of the animals was determined from an average computed by repeated weighings for several days before the actual start of the experiments. Subsequent change in the weight of the rabbits was not used as an indication for altering the dose.

The rabbits were bled before beginning inoculation and twice a week subsequently. The separated serum was kept in the ice box, and the sera obtained at several different dates were tested at one time against the homologous antigens. This precaution was taken so that the results might be as comparable as possible. In all except the first two or three experiments a single standardized pipette was used to dilute all of the sera in one series of tests; this pipette was washed five or six times with saline solution after making each dilution. In doing comparative work with dilutions of different sera this precaution was necessary, for the ordinary pipettes were found to vary from 10 to 20 per cent. After recording the strength of reaction in the ordinary way by a series of plus marks the antibody content was computed from an interpolation table and curve. In this way the strength of reaction can be recorded by means of curves or figures and the comparison made easier than when the reactions are merely recorded by a certain number of pluses.

In some of the earlier experiments the temperature of the rabbits was charted twice daily; subsequently this procedure was discontinued as it was found that the information obtained was of little value in interpreting the immunity results.

Influence of Salicylates on the Formation of Complement-Fixing Antibodies.

In the first three experiments the formation of complement-fixing antibodies against the homologous streptococci was followed.

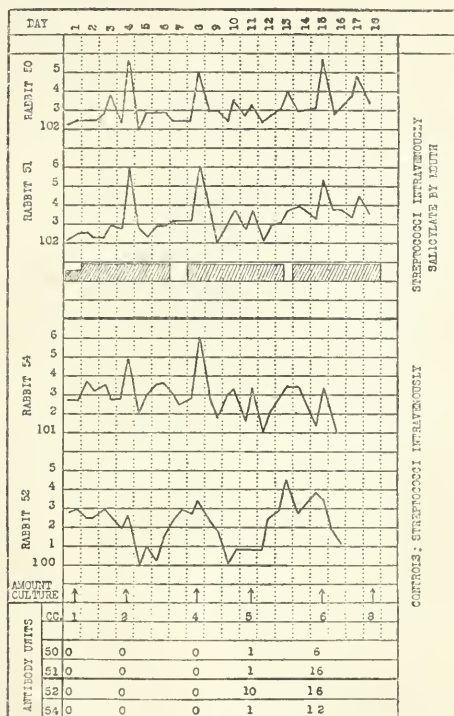
The antigens used in the tests were prepared by dissolving dried bacteria in antiformin and titrating this solution back to neutral according to the method described by Kinsella and Swift (3). The reactions were carried out in the usual manner, with a period of 1 hour for incubation of antigen, complement, and antibody; after which sensitized sheep red blood cells were added and the tubes returned to the water bath at 37°C. for 2 hours. Immediate readings were then made. The antigens were used in one-half the anticomplementary dose, and both the complement and hemolytic amboceptor in double the hemolytic dose.

Experiment 1.—Four rabbits of practically equal weight were chosen and weighed daily for a week; their temperatures were taken morning and evening as a control of the subsequent period. The animals were divided into two groups of two each. All received intravenous injections of living cultures of *Streptococcus viridans* A49 twice a week in amounts shown in Text-fig. 1. Rabbits 50 and 51 received daily sodium salicylate, 0.16 gm. per kilo of body weight. All of the sera were tested for complement-fixing antibodies on the same day with the same complement and antigen. The general results of the experiment are shown in Text-fig. 1.

It is evident that prolonged administration of sodium salicylate by stomach tube in the dose used had no deleterious effect on the rabbits. A loss of weight in all of the animals can be attributed to other factors than salicylates, such as the repeated inoculation with streptococci, or snuffles. Two control rabbits receiving the same amount of sodium salicylate, but not inoculated with streptococci, showed no loss in weight and little variation in the temperature curve.

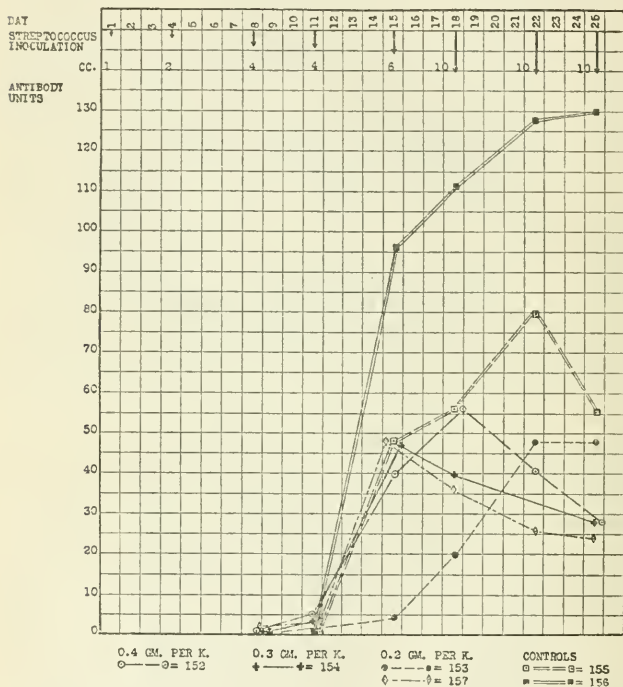
Apparently, the salicylates had little or no antipyretic effect, for the rabbits, whether receiving the drug or not, showed a similar fever within a few hours after intravenous inoculation of living streptococci. Rabbit 52 showed less effect following inoculation than did any of the others. The complement-fixing antibody is indicated in units. The first antibodies were detected in all of the rabbits on the 11th day following the first inoculation, when the two salicylated animals and one control showed equal titers; the other con-

trol had more antibodies. On the 15th day the combined non-salicylated controls yielded sera with stronger titer than did the salicylated animals. The experiment was terminated at this point because of the death of one of the controls.



TEXT-FIG. 1. Comparison of temperature curves and complement-binding antibody units in salicylated and control rabbits inoculated twice weekly with living *Streptococcus viridans* A49. The cross-hatching indicates periods of sodium salicylate administration.

Experiment 2.—Of six rabbits, two served as controls; the other four received sodium salicylate by stomach tube, two doses a day, as follows: No. 152, 0.4 gm. per kilo of body weight a day; No. 154, 0.3 gm.; Nos. 153 and 157, 0.2 gm. All of the animals were inoculated twice a week with increasing amounts of living



TEXT-FIG. 2. Comparison of complement-binding antibodies in rabbits receiving different amounts of sodium salicylate with controls; animals immunized twice weekly with living *Streptococcus viridans* A49.

Streptococcus viridans A49, as indicated in Text-fig. 2. Blood was obtained before each inoculation; the separated sera were stored until the termination of the experiment, and all were tested simultaneously against the homologous streptococcus for complement-fixing antibodies. The experiment was terminated after the 25th day; the results are shown in Text-fig. 2.

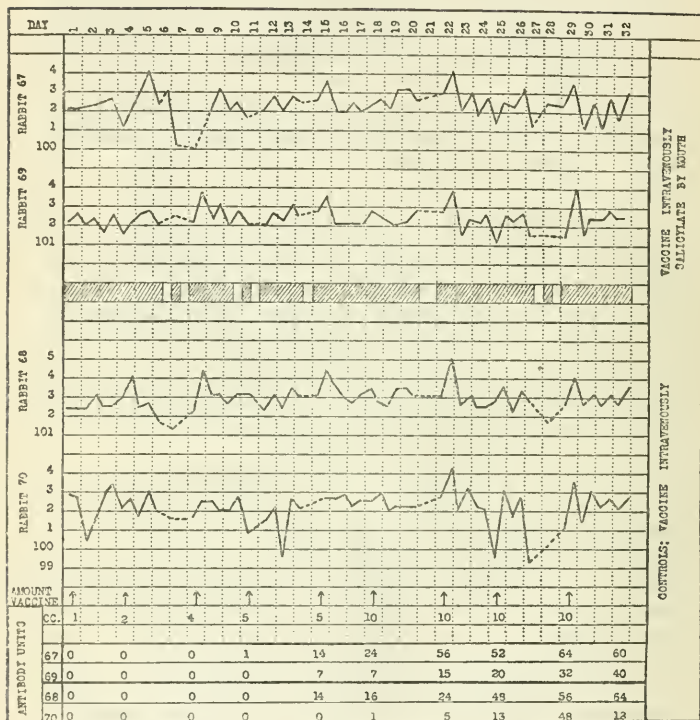
It is evident that there is no striking difference in the antibody content of the rabbits that received different amounts of sodium salicylate; the variation is no greater than might be seen in four different untreated rabbits. It is noteworthy that the two non-salicylated controls showed higher antibody curves than did any of the salicyl-treated animals.

All of the animals were weighed, and all showed similar losses of from 100 to 200 gm.; therefore, in so far as this feature is concerned, neither deleterious nor beneficial effects could be attributed to the salicylates.

Experiment 3.—The same general plan was followed as in Experiment 1, with the following exception. The rabbits were immunized with a stock vaccine prepared by suspending in normal saline solution the centrifugate of a 24 hour broth culture of hemolytic *Streptococcus K*, and killing the bacteria by heating at 56°C. for $\frac{1}{2}$ hour. The animals were immunized by injecting intravenously increasing amounts of this vaccine. Temperature and weight curves of the rabbits were followed; only the temperature curves are reproduced, as the weights of three of the animals increased during the experiment, and that of the fourth was stationary. Blood was withdrawn before each inoculation; the sera were tested at two different periods, after the 18th and 32nd days, respectively. The number of complement-fixing units is recorded in Text-fig. 3.

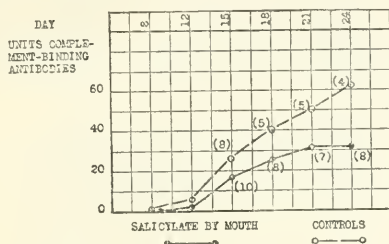
It is evident that there was little if any difference in the antibody content of the two groups of animals; the difference is no greater than might be expected in such small groups. The fever normally following the intravenous injections of bacterial vaccines was uninfluenced by the administration of salicylates in the same manner as was the fever following the intravenous inoculation of living *Streptococcus viridans*.

A similar experiment was conducted in which the rabbits were inoculated with living cultures of hemolytic *Streptococcus K*. These rabbits died from generalized streptococcus infection before sufficient time had elapsed to obtain comparable results. Here, again, no distinct antipyretic effect was seen in the salicyl-treated animals as compared with untreated controls. All four of the rabbits inoculated with living hemolytic streptococci had multiple purulent arthritis from which pure cultures of hemolytic streptococci were recovered. It is therefore evident that the salicylates had no influence in preventing the development of arthritis.



TEXT-FIG. 3. Comparison of temperature curves and complement-binding units in salicylated and control rabbits immunized twice weekly with vaccines of *Streptococcus hemolyticus* K. The cross-hatching indicates periods of sodium salicylate administration.

A summary of all of the experiments showing the influence of salicylates upon the formation of complement-fixing antibodies is given in Text-fig. 4. Here the averages derived from a larger group of animals than was used in any individual experiment indicates that salicylates in general had a depressing influence upon the formation of this type of immune bodies.



TEXT-FIG. 4. Comparison of average complement-binding antibody formation in the foregoing series of salicylated and control rabbits. The number of animals tested each day is indicated in parenthesis.

Influence of Salicylates on the Formation of Agglutinins.

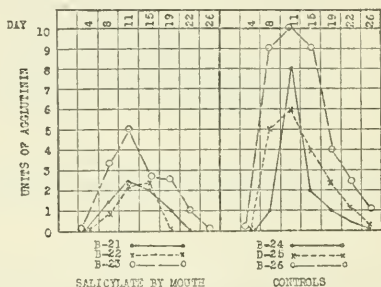
In the next four experiments the rate of agglutinin formation was determined.

In all experiments the sera were tested against killed cultures of the homologous bacteria prepared as follows: A 24 hour broth culture was heated at 56°C. $\frac{1}{2}$ hour, and standardized by Gates' (4) method so that the density of the suspensions was similar. The reaction of the suspensions was brought to a pH 7.3 or 7.4 to avoid acid agglutination. In order to insure that the suspending medium was the same in all of the tubes the sera were diluted with broth, instead of salt solution. The tubes were incubated at 56°C. for 2 hours, removed from the incubator, and allowed to cool for 15 minutes; then the strength of reaction was recorded.

Experiment 4.—Six brown rabbits weighing from 1,400 to 1,600 gm. were immunized with a pneumococcus of Type I by giving daily intravenous injections of vaccines prepared by suspending the centrifugate of 24 hour broth cultures in normal saline solution and heating at 56°C. for $\frac{1}{2}$ hour. An amount of vaccine representing 2 cc. of culture was given to each rabbit daily for 6 days. Three of the rabbits (Nos. B-21, B-22, and B-23) received sodium salicylate daily for

14 days; the dose was 0.16 gm. per kilo of body weight. The other three rabbits served as controls. The sera of all of the animals were tested for agglutinin content against the same suspension of homologous pneumococci. The results are given in Text-fig. 5.

In this experiment the amount of immunizing antigen was small and the period of immunization was short in comparison with the previous experiments. It is difficult to obtain in rabbits an agglutinating serum of high titer against pneumococci. In this series, therefore, the curves are low; the salicylated rabbits, nevertheless, had uniformly less antibodies than did the controls.



TEXT-FIG. 5. Comparison of agglutinin formation in salicylated and control rabbits immunized with six daily injections of pneumococcus vaccine.

Experiment 5.—Ten brown rabbits weighing between 1,700 and 1,800 gm. each were divided into two lots of five. One lot was given sodium salicylate, 0.16 gm. per kilo of body weight, daily for 20 days. On the 2nd day all of the animals received intravenously the centrifugalized sediment of 5 cc. of broth culture of *Streptococcus viridans* 38D; on the 6th day a similar amount of the same organism; and on the 14th day 10 cc. Blood was obtained from the animals before the beginning of treatment and on the 6th, 10th, 14th, 17th, and 21st days. Three of the salicylate-treated animals and one of the control group died during the course of the experiment; all of the surviving animals were killed and examined for lesions of the joints and viscera on the 20th or 21st day. At the termination of the experiment all of the sera were titrated for agglutinins. The final interpolated titers of the various sera are given in Table I.

TABLE I.
Comparison of Agglutinin Formation in Salicylated and Control Rabbits Inoculated with Living Streptococcus viridans 38D.

Date.	Day.	Inoculation.	Salicylated rabbits.							Controls.						
			A-52	A-54	A-56	A-58	A-60	A-62	Aver- age.	A-53	A-55	A-57	A-59	A-61	A-65	Aver- age.
1921 Apr. 2																
" 7	2	5 cc. of Streptococcus 38D.	0	0	0	0	0	0		0	0	0	0	0	0	
" 11	6															
" 11	6	5 cc. of Streptococcus 38D.	10	8	10	20	18	18	14	27	8	18	16	20	17	18
" 15	10															
" 18	10	10 cc. of Streptococcus 38D.	18	10	30	120	96	64	56	76	20	80	96	70	76	69
" 19	14															
" 22	17		224	176	256	480	320	Dead.	291	480	80	480	1,120	224	880	544
" 26	21		510	304	Dead.	512	560	"	471	640	152	512	1,200	Dead.	1,280	757
			510	640	"	1,200	Dead.	"	783	350	1,040	880	2,240	"	2,400	1,382

Although the usual variation in the agglutination titer in the serum of different animals is seen in both groups, most of the rabbits in the control group consistently yielded stronger agglutinating serum than did the salicyl-treated rabbits; the average curve of the control group was uniformly higher than that of the salicyl group, and on the 14th, 17th, and 21st days it was nearly twice as high.

In another experiment carried out in a manner similar to No. 5, part of the salicylated rabbits succumbed to intravenous inoculations of *Streptococcus* A49; on the 8th day the three surviving rabbits in this group gave an average agglutinin content of 46 units; and six controls gave an average content of 57 units. On the 16th day only a single salicylated animal was living, while five controls still survived; at this time both groups had an average agglutinin content of 900. Because of the difference in the number of animals in the two groups this experiment is not given in detail. It merely shows that a single salicylated animal may have as high an antibody curve as some untreated controls; and emphasizes the desirability of having more than one animal in each group.

In still another experiment in which only two salicylated rabbits and four controls lived until the 6th day, the average agglutinin curve on this day was 735 for the first and 450 for the second group. These animals were immunized with a single intravenous injection of 35 cc. of *Streptococcus viridans* A135. The difference in the number of animals in the two groups also decreases the value of the results in this experiment. It is possible that the large amount of immunizing antigen may have been a factor in eliminating the difference in the two groups of animals.

These two experiments were the only ones in which the animals receiving salicylates yielded serum with as high or higher average antibody content than that of control animals.

Experiment 6.—This experiment was designed to determine whether salicylate-treated rabbits and non-salicylated controls, all previously inoculated into the knee joints with killed cultures of green streptococci, would show any difference in the agglutinating power of their sera after intravenous inoculation with living streptococci.

Each of six brown rabbits weighing between 1,600 and 1,900 gm. was inoculated into the right knee with the sediment of 0.5 cc. of broth culture of *Streptococcus viridans* 38D killed by heating for $\frac{1}{2}$ hour at 56°C. and into the left knee with 0.5 cc. of *Streptococcus viridans* A49 similarly treated. The knees were measured and otherwise observed until they had reached their original dimensions or were stationary in size. Serum was separated from blood obtained on the 3rd, 20th, and 28th days following intraarticular inoculation. The animals were then divided into two groups of three each, and the members of one group given

sodium salicylate daily in doses of 0.2 gm. per kilo of body weight; this treatment was started on the 27th day after the intraarticular inoculation. The next day each of the animals received an intravenous injection of the sediment of living streptococci from 9.3 cc. of an 18 hour culture of *Streptococcus* 38D. Serum was obtained on the 3rd and 9th days following this intravenous inoculation, and on the 9th day all of the animals were autopsied to determine the extent of articular and visceral lesions.

In the immunity studies several points may be noted: (1) the possibility of detecting agglutinins in the blood following the intra-articular inoculation of small amounts of two different strains of killed streptococci; (2) the effect of subsequent intravenous inoculation of one of the two strains—whether it stimulates antibodies to one or both; and (3) the difference in the salicylate-treated and control groups. The results are summarized in Table II.

Although the antibody curve was not followed at frequent intervals after the intraarticular injection of killed streptococci, it is evident that a single injection of Strain A49 stimulated the formation of agglutinins much more than did a simultaneous injection of Strain 38D. It is impossible to state whether the agglutinins detected on the 28th day were stimulated by the intraarticular injection or by the intravenous inoculation because the blood was withdrawn 8 hours after the intravenous inoculation. The increase in agglutinins for Strain A49 on the 9th day after the intravenous injection of Strain 38D must have been due to the stimulating effect of the latter strain in animals already slightly immune to No. A49. The rise in antibody curve at this time was noted for both strains. The two strains are distinct antigenically; the serum of many animals immunized alone with Strain 38D has never agglutinated No. A49, and *vice versa*. The increase in antibodies for No. A49 on the last day must, therefore, have been a concomitant phenomenon, and did not seem to have been influenced by salicylates. The lower agglutinin content for *Streptococcus* 38D in the salicylated animals is similar to that observed in other experiments.

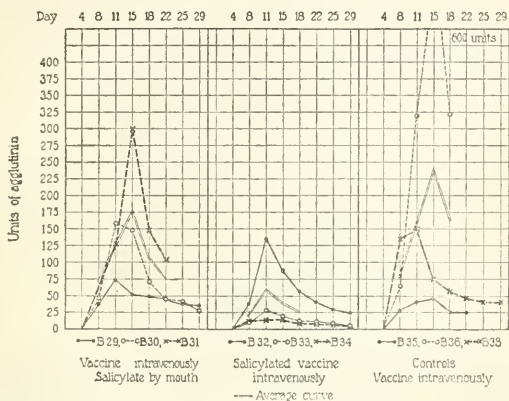
Experiment 7.—The object of this experiment was to determine in addition whether streptococci treated *in vitro* with salicylates were less active antigenically than untreated streptococci. Three groups of animals were chosen. Group 1, consisting of Rabbits B-29, B-30, and B-31, received sodium salicylate by stomach

TABLE II.

Comparison of Agglutinin Formation in Salicylated and Control Rabbits Inoculated with Streptococcus viridans 38D, but Previously Injected into the Knee Joints with Streptococcus viridans 38D and A49.

Date.	Day.	Inoculation.	Agglutination tested with Streptococcus 38D.						Agglutination tested with Streptococcus A49.							
			Salicylated rabbits.			Controls.			Salicylated rabbits.			Controls.				
			K-23	K-25	K-28	Aver- age.	K-22	K-24	K-26	Aver- age.	K-23	K-25	K-28	K-22	K-24	K-26
1921 Apr. 21	1	Right knee 0.5 cc. of killed Strepto- coccus 38D. Left knee 0.5 cc. of killed Strepto- coccus A49.	0	0	0		0	0	0		0	0	0	0	0	
" 23	3		0	0	1		0	0	1		1	0	0	2	1	1(?)
May 10	20		0	0	0		0	0	0		2	2	0	0	1	0
" 18	28															
" 18	1	9.3 cc. of living Streptococcus 38D intravenously.	0	0	0		0	0	1		2	0	1(?)	0	1(?)	0
" 20	3		480	140	480	366	300	720	560	526	8	2	4	2	4	4
" 26	9															

tube, 0.2 gm. per kilo of body weight, and daily injections of vaccine prepared from broth cultures of *Streptococcus viridans* 38D. The vaccine was given for 5 days. Group 2, consisting of Rabbits B-32, B-33, and B-34, received daily intravenous injections of salicylated vaccine. This vaccine was prepared as follows: An aliquot portion of the vaccine used in immunizing Group 1 was mixed with an amount of sodium salicylate solution that would have been used had the rabbit received the drug by stomach tube. The vaccine and salicylate were incubated at 37°C. for 4 hours, then centrifugalized, and the centrifugate was taken up in saline solution and injected intravenously. This vaccine was given for 5 days. Group 3, the control group, consisting of Rabbits B-35, B-36, and B-38, received



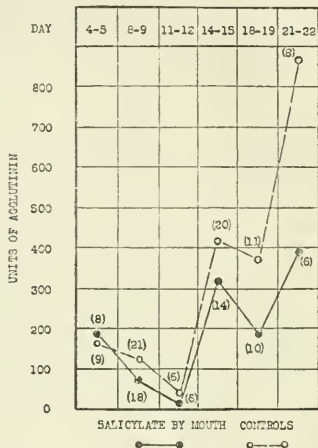
TEXT-FIG. 6. Comparison of agglutinin formation in salicylated and control rabbits immunized with vaccines of *Streptococcus viridans* 38D, and rabbits immunized with salicylated vaccines.

daily injections of untreated vaccine. All of the rabbits received each day vaccines in an amount equal to 2 cc. of broth culture per kilo of body weight. The rabbits were bled before the experiment and on other days indicated in Text-fig. 6. All of the sera were tested for agglutinin content on the same day. The results are shown in Text-fig. 6.

Here, as in previous experiments, the control group had a higher agglutinin content in their sera than did the rabbits treated with sodium salicylate. The second group, namely those that received salicylated vaccine, had the lowest antibody content. In this experi-

ment it seemed, therefore, that the action of the salicylate might be directly upon the antigen, causing it to be a less active stimulator of antibody production in the animal body.

It was thought advisable to determine whether streptococci treated with salicylate might act differently towards an agglutinating anti-



TEXT-FIG. 7. Comparison of average agglutinin formation in the complete series of salicylated and control rabbits. The number of animals tested each day is indicated in parenthesis.

serum than untreated streptococci. The following experiment was therefore performed.

A 24 hour broth culture of *Streptococcus* 38D was killed by heating $\frac{1}{2}$ hour to 56°C. The reaction of the medium at this time was pH 7.4. The culture was divided into two parts: X was kept in the original state; Y was treated as follows: It was centrifugalized and the supernatant serum broth retained. The separated bacterial sediment was mixed with 400 cc. of 5 per cent sodium salicylate and incubated for 4 hours at 37°C. It was then centrifugalized to clearness, the supernatant sodium salicylate solution discarded, and the bacterial sediment added to the original broth. The resuspension of the cocci in the medium in which they were grown insures that Suspensions X and Y were the same except that Suspension Y contained salicylated streptococci. These two suspensions were

then tested against the sera of Rabbits B-29, B-31, B-32, B-33, and B-34. With each serum the agglutination reached the same point in both suspensions; the differences in the agglutinating power of the serum of the rabbits of Groups 1 and 2 cannot, therefore, be attributed to the possibility that Group 2 rabbits were immunized with less agglutinable streptococci.

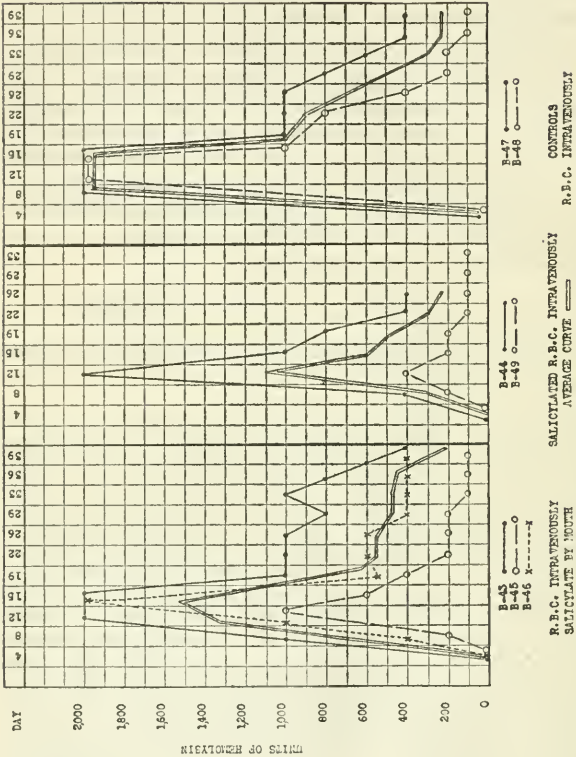
The average agglutinin content of the serum of all of the rabbits used in the foregoing experiments is shown in Text-fig. 7. The irregular course of the curves is due to the difference in the number of animals tested at different periods. At each period, however, the number of controls is equal to or greater than the number of salicylated animals. This summary shows that the average agglutinin formation in the entire group of salicylated animals was lower than that of the controls, and is in accord with the findings of the animals tested for complement-binding antibodies.

Influence of Salicylates on the Formation of Hemolysin.

In the remaining experiments the antibody tested was an hemolysin against sheep red blood cells.

All of the sera were inactivated by heating at 56°C. for $\frac{1}{2}$ hour. The diluted serum was then mixed with guinea pig serum so diluted that 0.5 cc. equaled one unit of complement previously tested with a constant standard hemolytic serum. To the rabbit serum and guinea pig complement was added 0.5 cc. of a 5 per cent suspension of sheep red blood cells. The mixtures were incubated in the water bath at 37°C. for 2 hours and read immediately. The dilution at which hemolysis was complete was considered as containing the charted unit of antibody. Because of the result obtained in Experiment 7 the experiments with hemolysins were all carried out with three groups of animals: Group 1, in which the animals received washed sheep red blood cells intravenously and sodium salicylate by stomach tube; Group 2, in which the animals received intravenous injections of salicylated sheep red blood cells, prepared by incubating together washed erythrocytes and a 2.5 per cent sodium salicylate solution, then centrifugalizing the mixture and retaining the cells alone for inoculation; Group 3, in which the animals received simply intravenous injections of washed sheep red blood cells.

Experiment 8.—Seven rabbits about 6 weeks old, all brown, of the same litter, were chosen for this experiment. Inactivated serum obtained from a preliminary bleeding was tested for hemolysin content against sheep red blood cells. The rabbits were then divided into three groups according to their weight and the native hemolysin content of their serum. This precaution was taken because certain investigators claim that the amount of antibody formation following



TEXT-FIG. 8. Comparison of hemolysin formation in salicylated and control rabbits immunized with sheep erythrocytes, and rabbits immunized with salicylated erythrocytes. Salicylate and erythrocytes given daily for 3 days, 1 day intermission, then for 3 more days.

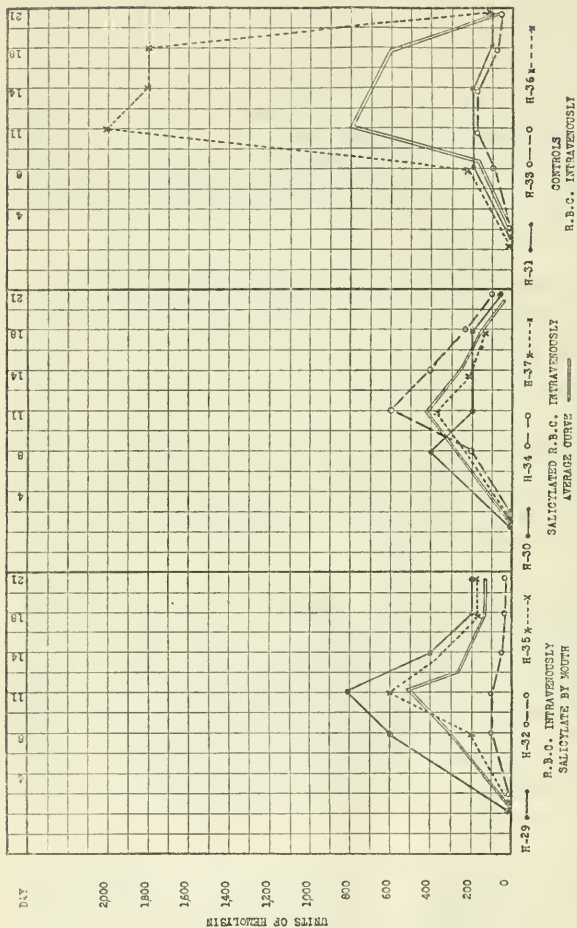
immunization is more or less in direct proportion to the amount of antibody the serum of the animal contained before immunization. Group 1, consisting of Rabbits B-43, B-45, and B-46, received injections of sheep cells daily for 3 days followed by an interval of 1 day, and then for 3 more days, and at the same time sodium salicylate by stomach tube—a total daily amount of 0.16 gm. per kilo of body weight per day. Group 2, consisting of Rabbits B-44 and B-49, received intravenous injections on the same days as did rabbits of Group 1. They received sheep red blood cells that had been incubated for 3 hours with sodium salicylate, the supernatant salicylate solution discarded, and the salicylated cells resuspended in saline solution. Group 3, controls, consisting of Rabbits B-47 and B-48, was injected with washed sheep blood cells. The antibody curves of these rabbits are shown in Text-fig. 8.

In all three groups some of the rabbits showed hemolysin titers as high as 1,000 to 2,000. Comparing the average curve for each group, however, the highest and most prolonged antibody content was found in Group 3, the next in Group 1, and the lowest in Group 2, the group immunized with salicylated cells.

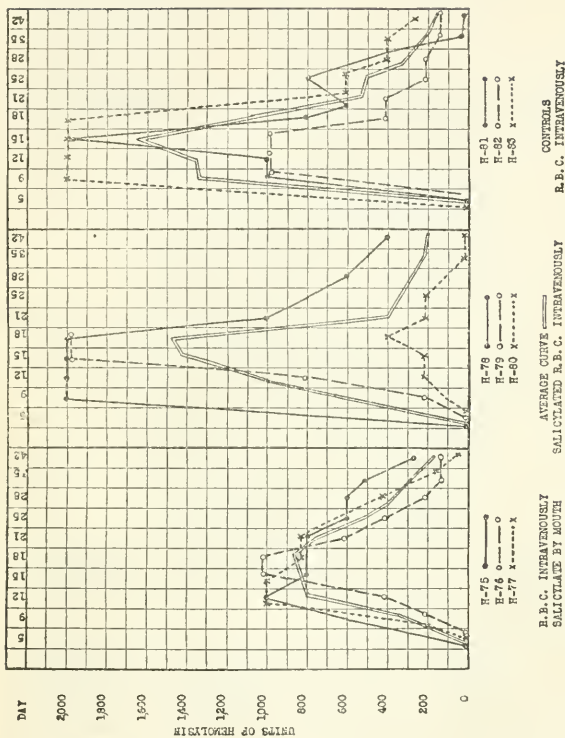
Experiment 9.—This experiment was carried out in essentially the same manner as Experiment 8, with the following exceptions. Three groups of larger rabbits, consisting of three animals each, were used; injections of erythrocytes were given only 3 days; and the sodium salicylate by mouth was given for a similar period. The influence of the salicylate was, therefore, correspondingly less than in animals receiving the drug over a longer period. The results are shown in Text-fig. 9.

In this experiment the differences between the various groups were less marked than in previous experiments although the average curve in the control group (Group 3) was higher than in Groups 1 and 2. This high average is due to the fact that the serum of Rabbit H-36 had a much higher antibody content than that of any of the others. The other two rabbits in this group had a lower antibody content than any of the rabbits in the other two groups. As in Experiment 8 the animals receiving salicylate by stomach tube had a higher average curve than did those receiving salicylated red blood cells intravenously.

Experiment 10.—A comparison of the results in Experiments 8 and 9 seems to indicate that a long or short period of administration of the salicylates might be a factor in determining the degree of antibody formation. Experiment 10 was made to test the influence of more prolonged administration of the drug. The rabbits used in this experiment were all brown, full grown, and of approximately

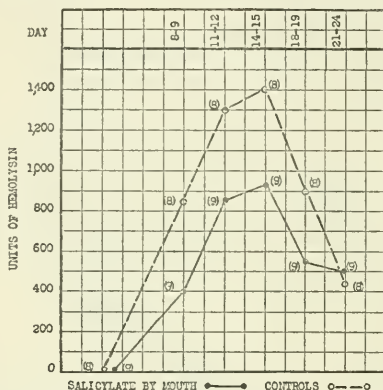


TEXT-FIG. 9. Comparison of hemolysin formation in salicylated and control rabbits immunized with sheep erythrocytes, and rabbits immunized with salicylated erythrocytes. Salicylate and erythrocytes given daily for 3 days.



TEXT-FIG. 10. Comparison of hemolysis formation in salicylated and control rabbits immunized with sheep erythrocytes and rabbits immunized with salicylated erythrocytes. Salicylate given for 12 days; erythrocytes injected on the 1st, 5th, and 9th days.

the same size. Three intravenous injections of 25 per cent sheep red blood cells, 4 cc. per kilo of body weight, were given to each rabbit on the 1st, 5th, and 9th days, respectively. In Groups 1 and 3 the cells were not salicylated; in Group 2 the cells were salicylated by incubating them with 2.5 per cent sodium salicylate solution for 15 hours, the suspension centrifugalized, the supernatant solution discarded, and the cells resuspended in saline solution. Group 1 animals received sodium salicylate in a total daily amount of 0.2 gm. per kilo of body weight. The animals received three doses before the first immunizing cells were given and were treated 4 days after the last intravenous injection of erythrocytes. The results are shown in Text-fig. 10.



TEXT-FIG. 11. Comparison of average hemolysin formation in the salicylated and control rabbits used in Experiments 8 to 10. The number of rabbits tested each day is indicated in parenthesis.

Here, as in previous experiments, the control group had a higher total as well as higher average antibody content than did the other two groups. Group 1, in which the rabbits received salicylate for 12 days, showed the lowest antibody content. Group 2, treated with salicylated cells, showed almost as great an antibody content as did Group 3. The discrepancy between the animals in Group 2 in this experiment and in the three previous experiments cannot be explained.

If the average hemolysin content of the sera of all of the animals used in Experiments 8 to 10 are plotted (Text-fig. 11), it is seen that

the control animals not receiving salicylate yielded sera with consistently higher titer than did animals receiving salicylate by mouth. This is in complete accord with the results obtained for agglutinating and complement-binding antibodies.

DISCUSSION.

Aside from the two incomplete experiments in which the number of animals in the salicylated group was small, the controls all had a higher antibody content than did animals similarly immunized but treated with sodium salicylate. The dose of salicylate given the rabbits each day was comparable with the largest amount that may be safely administered to patients for short periods, and much larger than can be tolerated over a long period by patients with rheumatic fever. While some rabbits receiving 0.4 gm. per kilo each day seemed to suffer no deleterious effects, others receiving this amount of the drug succumbed more quickly to intravenous inoculation of living streptococci than did unsalicylated controls.

Fantus, Simmonds, and Moore (5) showed that sodium salicylate, when used in a dose which they state was comparatively harmless to animal controls, is decidedly detrimental and liable to be fatal to rabbits infected with hemolytic streptococci. In their experiments from 0.9 to 1 gm. of sodium salicylate and 1.2 gm. of acetylsalicylic acid were given by mouth each day; in another series 0.3 gm. of sodium salicylate was given hypodermically. These are much larger doses than may be given with safety to patients. Our experiments indicate that 0.2 gm. is as large a daily dose as can be safely administered to rabbits over long periods.

Rabbits receiving the drug daily by stomach tube at first refused to take much food, but after 3 or 4 days they again resumed their normal feeding habits. Malnutrition in this group of animals cannot, therefore, be considered as the cause of a lowered antibody formation.

It is possible that animals with less febrile response following the intravenous injection of antigens might show a correspondingly lower antibody formation. As the salicylates have a marked antipyretic effect in patients this argument might be used theoretically to explain our results. In the first and third experiments it was shown, however, that salicylates in the doses employed did not have an anti-

pyretic effect in inoculated rabbits. A hypothetical correlation between fever and antibody formation must, therefore, be abandoned in explaining the results.

Only a few drugs or chemicals have been tested to determine their action upon the formation of immune bodies.

With the introduction of salvarsan several workers (6, 7) claimed that one of the marked actions of arsenicals in general was to increase antibodies. Others (8, 9) have claimed that this effect was less marked and less constant than found by the earlier experimenters. Toyama and Kolmer (9) state that large doses of both salvarsan and mercury bichloride appear to depress hemolysin and agglutinin production in immunized rabbits; while smaller doses of both drugs tend to increase the production of hemagglutinins, but not of hemolysins.

Müller (10) found that animals treated with aleuronat or cinnamic acid while undergoing immunization yielded more antibodies than did controls. He thought that because these two substances stimulated leucocytosis the two effects were to be attributed to a common action. Similarly the depression in immune bodies in animals under the influence of benzene led Hektoen (11) to believe that substances exerting a destructive action on the blood-forming organs had a depressing effect on the tissues from which immune bodies arise. But his later studies concerning the effect of toluene (12) and thorium (13) on immune body production indicate that variations in the antibody content of the blood serum and leucocytosis or leucopenia need not necessarily go hand in hand.

Abbott and Bergey (14), Müller (10), and Friedberger (15) have all demonstrated conclusively that alcohol in repeated doses has a very depressing influence on the capacity of immunized animals to form antibodies. Melnikowa and Wersilowa (16), and Müller (10) have shown that phlorhizin has a similar effect. Hektoen and Corper (17) have shown that mustard gas (dichloroethylsulfide) depresses immune body formation.

The foregoing review of previous experiments fails to furnish any explanation that can be brought to bear upon our studies.

Cook (18) has shown that the rate of antibody formation is dependent upon the rate of absorption of the antigen. She reviews and makes use of the work of Loeb, Lillie, and Osterhout, all of whom have demonstrated that the permeability of cells is increased by increasing the sodium ions and decreased by increasing the calcium ions. In her experiments, rabbits treated with sodium citrate formed antibodies more intensely than did controls, and those treated with calcium chloride less intensely than controls. In all of our experiments the rabbits receiving salicylates were at the same time subject to an

increased amount of sodium from the sodium salicylate. From Cook's experiments one would expect an increased antibody output if the sodium ion is the important element in the compound. Whether the salicyl ion has an antagonistic effect by decreasing the absorption of antigen is a question that must be submitted to experimental study. Reasoning simply by similarity of effect it seems possible that the decreased antibody production in rabbits treated with sodium salicylate may be due to the decreased power of these animals to absorb antigen.

It must be emphasized in this connection that there is nothing in our experimental results to indicate that the salicylates are deleterious in infection simply because they decrease immune body formation. In none of the experiments was this decrease extremely marked, and all of the salicylated animals produced immune bodies in fair amounts. It is probable that immune bodies in the circulating fluids are not the only factors concerned in the process of recovery. The same mechanism that makes an infectious agent less antigenic might at the same time make it less pathogenic. It is possible that this effect may be more marked in rheumatic fever than in other infections; hence the more striking effect of the drug seen in this disease.

CONCLUSION.

1. Rabbits treated with sodium salicylate in daily doses of from 0.16 to 0.2 gm. per kilo of body weight and at the same time immunized with intravenous injections of *Streptococcus viridans*, both living and in the form of vaccines, and also with washed sheep red blood cells, showed diminished complement-fixing antibodies, agglutinins, and hemolysins when compared with controls similarly immunized.

2. If the antigens were treated with sodium salicylate *in vitro* and subsequently injected intravenously into rabbits, the animals usually showed lower antibody curves than did rabbits that received the untreated antigen intravenously and sodium salicylate by stomach tube.

3. The beneficial effect of sodium salicylate in rheumatic fever patients probably cannot be attributed to an increased production

of circulating immune bodies against the infectious agent. This is, however, no contraindication to the administration of salicylates to patients suffering from infectious diseases.

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